Detection of *In Vitro* and *In Vivo* Oxidative Modifications of Ferritin and Transferrin by Mass Spectrometry: Hereditary Hemochromatosis As A Model

A Thesis

Submitted to the College of Graduate Studies and Research

For the Degree of

MASTER OF SCIENCE

In the Department of Pathology and Laboratory Medicine,

Division of Clinical Chemistry

College of Medicine

University of Saskatchewan

By

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ABSTRACT

Hereditary Hemochromatosis (HH) is an inherited recessive autosomal disorder characterized by accumulation of excess iron. When iron binding proteins become saturated, concentrations of free, or non-transferrin-bound iron (NTBI) rise, a condition thought to be responsible for the adverse effects associated with HH. To investigate that disturbing iron homeostasis plays a role in free radical injury in HH, protein carbonyls were found to be 1-7 times higher in patients with HH than in controls, with the greatest increases being observed in untreated HH patients with high ferritin and >90% transferrin saturation with iron. An Unpaired *t* test revealed a P value of 0.0278 ($P < 0.05$), which is considered to be statistically significant. Our data showed a significant positive correlation (linear relationship) between the level of carbonyl content and ferritin concentration in plasma samples from patients with HH. *In vitro* oxidation of transferrin and ferritin standards with hydrogen peroxide and excess iron, followed by immobilized trypsin digestion (Poroszyme), high-resolution LC-MS/MS analysis (Q-TOF Ultima, Waters) and MS/MS data processing (PEAKS, Bioinformatics Solution), identified several tryptic peptides containing oxidized Met, Trp and His residues. Mapping of the oxidized ferritin residues showed them to be located on the inner face of each sub-unit, the face directed toward the ferritin core where iron is normally stored. Using the same methodology, oxidized residues were subsequently detected in ferritin and transferrin isolated from plasma samples of patients severely affected with HH. Comparing of MS/MS spectra of *in vitro* oxidized samples that have most fragment ion peaks in common with oxidized peptide MS/MS spectra from samples of patients with HH revealed a significant correlation between the two. These data show that elevated NTBI may be involved in oxidative modification of the iron binding proteins, ferritin and transferrin, and that such modifications may play a significant role in the pathophysiology of HH.

ACKNOWLEDGMENTS

I wish to express my sincere appreciation and deep sense of gratitude to my supervisor Dr. Denis Lehotay for his unending guidance, encouragement and personal concern throughout the course of my research project. I also acknowledge and appreciate the helpful suggestions, concerns and guidance given by the advisory committee members; Dr. John Krahn, Dr. Andrew Ross, Dr. Scott Napper, Prof. Mabood Qureshi.

I would also like to take the opportunity to thank Mr. Douglas Olson at the NRC- PBI Mass Spectrometry Lab for his help throughout the course of this research project. My sincere thanks to Ms. Heather Neufeld and Mr. Todd Reichert for their help during the preparations for seminars and other technical problems.

I extend my sincere thanks and appreciation to my family for their support, co-operation, and patience through the course of my research project. In the end I would like thank everybody who supported me if I have forgotten to mention their names.

To my wife, Eman

and my children; Anan, Ahmed and Sarrah

for their support

TABLE OF CONTENTS

REQUEST FOR PERMISSION i
ABSTRACT ii
ACKNOWLEDGMENTS iii
DEDICATION iv
TABLE OF CONTENTS v
LIST OF TABLES viii
LIST OF FIGURES x
ABBREVIATIONS xii
1.0 Introduction 1
1.2 Is oxidative modification reversible? 3
1.3 Advantages of protein oxidation as a biomarker for oxidative stress 4
1.4 Radical-mediated protein oxidation 4
1.4.1 Transition Metals 5
1.4.2 Fenton Reaction 5
1.5 Non-radical mediated protein oxidation 5
1.6 An Overview for Oxidative Modifications of Different Amino Acids 6
1.6.1 Oxidative Modification of Protein Thiols 6
1.6.2 Oxidative Modification of Tyrosine 7
1.6.3 Oxidative Modification of Methionine 8
1.6.4 Protein Carbonylation 9
1.6.5 Oxidation Of Tryptophan 10
1.6.6 Oxidation of Histidine 11
1.7 Study of Ferritin and Transferrin as a Model of Oxidative Modifications of Proteins 12
1.7.1 Ferritin 12
1.7.2 Transferrin 14
1.8 Hydrogen Peroxide (H_2O_2) As an Oxidizing Agent in Biological Systems 17
1.9 Mass Spectrometry As an Analytical Technology 19
1.9.1 Key Technologies In The Study of Proteins 21
1.10 Hereditary Hemochromatosis 31
1.10.1 History 31
1.10.2 HFE gene and protein 31
1.10.3 Pathophysiology of HH 33
1.10.4 Diagnostic criteria and treatment 34
2.0 Hypothesis 36
3.0 Rationale 36
4.0 Objective of the Study 36
5.0 Materials and Methods 37
5.1 'Gel-free' Protein Digest Experiment 37
5.1.1 Poroszyme™ Method for Protein Digestion 37
5.1.2 MALDI-TOF MS 38
5.2 In Vitro Oxidation of Ferritin and Transferrin Experiments 40
5.2.1 Poroszyme method for Protein Digestion 41
5.2.2 Protein Identification by Q-TOF LC-MS/MS: 41
5.3 In Vitro Excess Iron Overload and Oxidation of Ferritin 43

5.4 In Vitro Excess Iron Overload and Oxidation of Transferrin 44
5.4.1 Experimental Design 44
5.5 In Vivo Detection of Oxidative Modification In Plasma Samples of Patients of .....
Hereditary Hemochromatosis (HH) 45
5.5.1 Immunoprecipitation Protocol: 45
5.5.2 SDS-Polyacrelamide Gel Electrophoresis (Linear Slab Gel) 46
5.5.4 Analysis using MALDI-TOF and Q-TOF LC-MS/MS 49
5.6 Specrophotometric Assay for the Detection of Protein Carbonyls in the Plasma .....
Samples of Patients With Hereditary Hemochromatosis 50
5.6.1 Assay Range 50
5.6.2 Pre-Assay Preparation and Stock Solutions 50
5.6.3 Plasma Samples Preparation 50
5.6.4 Procedure 51
5.6.5 Determination of Carbonyl Content 52
6.0 Results 53
6.1 Results from 'gel-free' protein digest experiments 53
6.2 Oxidized and unmodified peptides matching human transferrin and ferritin 56
6.2.1 Transferrin 57
6.2.2 Ferritin 59
6.2.3 Oxidized peptides that provided significant matches with human .....
transferrin (i.e. targets for LC-MS/MS analysis) 61
6.3 Database search results for ferritin and transferrin LC-MS/MS data processed .....
using PEAKS 64
6.4 Oxidized peptides identified during in vitro experiments and selected for .....
inclusion and targeted Q-TOF LC-MS/MS analysis of biological samples 75
6.5 1-D SDS-PAGE for plasma samples of patients of HH 76
6.6 Summary of PEAKS database search results using data from targeted LC-MS/.....
MS analysis of in previous in vitro experiments of oxidized peptides 78
6.7 Detection of protein carbonyls in plasma samples of patients with HH 89
7.0 Discussion 93
7.1 Protein Carbonylation and Lipid Peroxidation as Biomarkers of Oxidative Stress 93
7.2 Why Hydrogen Peroxide? 98
7.3 Autoxidation and Iron Incorporation in Ferritin Molecules 101
7.3.1 Ferritin oxidation and ·OH production 102
7.4 Autoxidation and Iron Incorporation in Transferrin Molecules 104
7.4.1 Does transferrin–bound iron affect lipid peroxidation? 107
7.4.2 Is Iron-loaded Transferrin able to generate ·OH? 108
7.5 Methionine Oxidation 110
7.5.1 Significance of In Vitro Met Oxidation 110
7.5.2 Is there a relationship between methionine and tryptophan oxidation? 113
7.6 Tryptophan Oxidative Modification as a Biomarker of Oxidative Stress 114
8.0 Conclusions 116
9.0 References 117

LIST OF TABLES **PAGE**

Table.1. Oxidative modifications of proteins……3

Table.2. The different concentrations of H_2O_2 and
the time of exposure of ferritin and transferrin.
to oxidative stress……40

Table.3. Comparison of control and HH patients……..51

Table.4. An example of oxidized and unmodified
peptides matching human transferrin……..57

Table.5. An example of oxidized and unmodified
peptides matching horse spleen ferritin……59

Table.6. Summary of the significant matches with
human transferrin including only the
.oxidized peptides from all samples……..61

Table.7. Summary of oxidized peptides that
provided significant matches with horse
spleen ferritin including only the oxidized
peptides from all samples……63

Table.8. A list of native ferritin peptides oxidized
with 20 µM H_2O_2……65

Table.9. A list of denatured ferritin peptides oxidized
with 20 µM H_2O_2……65

Table.10. A list of native transferrin peptides oxidized
with 20 µM H_2O_2……..66

Table.11. A list of denatured transferrin peptides
oxidized with 20 µM H_2O_2.....69

Table.12. Different modified and unmodified of protein
digest of plasma sample 2 of patient with HH……79

Table.13. Different modified and unmodified of protein
digest of plasma sample P2A of patient with HH……82

Table.14. Different modified and unmodified of protein
digest of plasma sample P4A of patient with HH……84

Table.15. Different modified and unmodified of protein

digest of plasma sample P6 of patient with HH……...86

LIST OF FIGURES **PAGE**

Fig.1.Schematic diagram of Voyager-DE STR MALDI-TOF mass spectrometer (Applied biosystems)……20

Fig.2. Mechanism of desorption and ionization in MALDI…….23

Fig.3. Schematic diagram of a tandem mass spectrometer…….25

Fig.4.The formation of charged droplets by Electrospray…….29

Fig.5. MALDI-TOFMS spectra of immobilized trypsin (Poroszyme) digests for (A) Transferrin (6μg) and (B) Ferritin (60 ng)……..54, 55

Fig.6. MS/MS spectrum of *m/z* 606.3192 from *in vitro* sample MOH070505_002 annotated by PEAKS after database matching against transferrin (Accession gi|31415705; 73.2% confidence)……..62

Fig.7. MS/MS spectrum of *m/z* 747.9221 from *in vitro* sample DF-3, annotated by PEAKS after database matching against transferrin (Accession gi|62897069; 99% confidence)…….67

Fig 8.MS/MS spectrum of *m/z* 797.5536 from *in vitro* sample NT-4, annotated by PEAKS after databas matching against transferrin (Accession gi|15021381; 65.3% confidence)…….68

Fig.9. MS/MS spectrum of *m/z* 633.4001 from *in vitro* sample DT-4, annotated by PEAKS after database matching against transferrin (Accession gi|15021381; 99% confidence)…….....70

Fig.10. 3-Dimensional structure of Horse Spleen Ferritin . Oxidized Met residues (yellow) are located on the inner face of each sub-unit……72

Fig.11. 3-Dimensional structure of Horse Spleen Ferritin . Oxidized Trp residues (yellow) are located on the inner face of each sub-unit……73

Fig.12. 3-Dimensional structure of Horse Spleen Ferritin . Oxidized His residues (yellow) are located on the inner face of each sub-unit……74

Fig.13. 1-D SDS-PAGE shows transferrin bands (Trf), visualized by Coomassie stain at the molecular mass of 78 kDa from plasma samples of patients with HH……..77

Fig.14. Annotated MS/MS spectrum for the peptide SASDLT(OxW)DNLK (*m/z* 633.1517)…….....80

Fig.15. Annotated MS/MS spectrum for the peptide TAGWNIP(OxM)GLLYNK (*m/z* 797.5013)…….81

Fig.16. Annotated MS/MS spectrum for the peptide DSGFQ(OxM)NQLR *(m/z* 606.30426)……83

Fig.17. Annotated MS/MS spectrum for the peptide CLVDLN(KyW)NNLEGK (*m/z* 761.4223)……...84

Fig.18. MS/MS spectrum of *m/z* 761.4239 from tissue sample P4A, annotated by PEAKS after database matching against transferrin (Accession gi|62897069; 88.2% confidence)…….85

Fig.19. Carbonyl groups, an index of oxidative stress, are increased in HH patients……..91

Fig.20. Carbonyl content vs ferritin……...92

Fig.21.Methionine and its oxidation products…….111

ABBREVIATIONS

ROS	Reactive Oxygen Species
O^{-}_{2}	Superoxide Anion
H_2O_2	Hydrogen Peroxide
·OH	Hydroxyl Radical
SOD	Superoxide Dismutase
Fe(II)	Ferrous Ion
Fe(III)	Ferric Ion
Cu(I)	Cuprous Ion
HOCl	Hypochlorus Acid
Cys	Cysteine
Met	Methionine
Lys	Lysine
Arg	Arginine
Pro	Proline
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosin
GSH	Reduced Glutathione
His	Histidine
Val	Valine
MCO	Metal-catalyzed Oxidation
MDA	Malondialdehyde
LDL	Low Density Lipoprotein
·ON	Peroxynitrite
HO_{2}·	Hydroperoxyl Radical
ROO·	Peroxyl
RO·	Alkoxyl
Mn	Manganese
Zn	Zinc
DOPA	Dihydroxyphenylalanine
SDS-PAGE	Sodium Dodecyl Polyacrylamide Gel Electrophoresis
ESI-MS/MS	Electrospray Ionization Tandem Mass Spectrometry
LC-ESI/MS	Liquid Chromatography Tandem Mass Spectrometry
GC-MS	Gas Chromatography Mass Spectrometry
MALDI-MS	Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry
CID	Collision Induced Dissociation
4-HNE	4-Hydroxynonenal
DNPH	2,4-dinitrophenylhydrazine
ELISA	Enzyme Linked Immuno Sorbent Assay
HTRP	Hydroxytryptophans
NFK	N-formylkynurenine
KYN	Kynurenine
3OH–KYN	3-Hydroxykynurenine
HPLC	High Performance Liquid Chrmomatography

TOF	Time-of-Flight
Q-TOF	Quadrupole Time-of-flight
Trf	Transferrin
TfR	Transferrin Receptor
NTBI	Nontransferrin-bound iron
MPO	Myeloperoxidase
PMA	Myristate acetate
PMM	Peptide Mass Mapping
DHB	Dihydroxybenzoic acid
m/z	Mass to charge ratio
HH	Hereditary Hemochromatosis
β_2M	β_2-microglobulin
Heph	Hephaestin
UIBC	Unsaturated Iron Binding Capacity
TIBC	Total Iron Binding Capacity
TFA	Trifloroacetic Acid
TCA	Trichloroacetic Acid
EDTA	Ethylenediaminetetraacetic Acid
DPTA	Diethylenetriaminepentaacetic Acid
NTA	Nitriloltriacetic Acid
$FeCl_3$	Ferric Chloride
FNA_{c3}	Ferric Nitrilotriacetate
NaCl	Sodium Chloride
PBS	Phosphate-buffered saline
BSA	Bovine serum albumin
PBST	PBS + Tween 20
DDT	Dithiothreitol
HCl	Hydrochloric acid
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
HCV	Hepatitis C Virus
DMT1	Divalent metal transporter

1.0 Introduction

Protein oxidation is the covalent modification of a protein induced either directly by reactive oxygen species (ROS) or indirectly by reaction with secondary by-products of oxidative stress (Shacter, 2000). Mitochondria are the major source of cellular ROS in non-phagocytic cells. Toxic oxygen metabolites are physiologically generated at a low level in cells and tissues during oxidative phosphorylation (Stadtman, E. et al. 1998). The resulting low levels of ROS play an integral part in modulation of several physiological functions of the cell, including gene expression, signal transduction, and defense against invading pathogens (Stadman, E. et al. 1998). ROS have the ability to damage all biomolecules including proteins, lipids, DNA and carbohydrates. ROS such as superoxide anion ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\cdot OH$) are formed in the course of normal metabolism through leakage of electrons from the electron transport chain and by activities of oxidoreductase enzymes such as NADPH oxidase, myeloperoxidase, xanthine oxidase, glucose oxidase, the cytochrome P450 and cyclooxygenases (Shacter, 2000). Under normal conditions, up to 1% of the mitochondrial electron flow leads primarily to the formation of $O_2^{-}\cdot$. Interference with electron transport can dramatically increase $O_2^{-}\cdot$ production, which is rapidly converted within the cell to H_2O_2 and O_2 by the superoxide dismutases (SODs). H_2O_2 can react with reduced transition metals [Fe(II), Cu (I)] via the Fenton reaction to produce the highly reactive hydroxyl radical. Alternatively, H_2O_2 may be converted into water by the enzyme catalase and glutathione peroxidase (Stadtman, E. et al. 1998). The modification of proteins is initiated mainly by the reactions with $\cdot OH$; however, the course of the oxidation process is determined by the availability of O_2 and $O_2^{-}\cdot$.

Collectively, ROS can lead to oxidation of amino acid residue side chains, formation of protein-protein cross-linkages and oxidation of the protein backbone resulting in protein fragmentation (Dean, R. et al., 1997). In addition, some other agents are involved in initiating protein oxidation such as HOCl (Shacter, 2000; Heinecke J. et al. 1993; Schraufstatter, I. et al. 1990; Handelman, G. et al., 1998; Yang, C. et al. 1997; Yan, L.et al. 1996), xenobiotics such as paraquat (Korbashi, P. et al., 1986; Hartley, D. et al. 1997), acetaminophen (Tirmenstein, M. et al., 1990), cigarette smoke (Eiserich, J. et al. 1995), γ-irradiation in the presence of O_2 (Berlett, B. et al. 1997; Steinbrecher, U. et al. 1987; Yan, L. et al. 1997), activated neutrophils (Davies, K. et al. 1987), UV light (Davies, K. et al. 1987; Fu,S. et al. 1997; Oliver, C. et al. 1987), ozone (Balasubramanian, D. et al. 1990), and by-products of lipids and free amino acid oxidation (Shen, H. et al. 1996; Hu, M. et al. 1992; Cross, C. et al. 1992). Due to the oxidative modification of many amino acid side chains, there are numerous types of protein modifications. The two amino acids that are especially prone to oxidative attack are Cys (Shacter, 2000; Radi, R.et al. 1991; Lii, C-K. et al. 1994) and Met (Shacter, 2000, Vogt, W. 1995), both of which contain susceptible sulfur containing amino acids (thiols). Indeed, most if not all ROS can induce modifications of Cys and Met. In the case of Cys, oxidation leads to the formation of disulfide bonds, mixed disulfides and thiyl radicals (Hu, M. et al. 1994; Kalyanaraman, B. et al. 1995). With Met residues, the major product under biological conditions is Met sulfoxide (Volt, W. 1995). Other amino acid moieties, especially Lys, Arg, Pro, and Thr (Shacter, 2000; Amici, A. et al. 1989) participate in the formation of carbonyl groups (aldehydes and ketones) on the side chains. The interaction of HOCl with Tyr, Trp, Lys and Met residues leads to formation of chlorotyrosine, chloramines, aldehydes and Met sulfoxides (Hazell, L. 1994; Hazen, S. et al 1997). Additional aspects of the oxidative modification of the protein will be reviewed in a subsequent section.

Protein oxidative modifications are summarized in Table 1. Because of the potential of ROS to induce significant biological damage, cells and tissues have an abundance of antioxidant systems to scavenge or eliminate them. These include antioxidant enzymes such as catalase, superoxide dismutases and glutathione peroxidase as well as low molecular weight scavengers such as vitamins C and E as well as reduced glutathione (GSH). Under normal conditions there is a well managed balance between formation and neutralization of ROS, so there is little or no modification of biomolecules. However, " oxidative stress " occurs when the balance of formation of oxidants exceeds the ability of antioxidant systems to remove or scavenge ROS (Shacter, 2000).

Table 1: Oxidative Modifications of Proteins

Modification	Amino acid involved	Oxidizing source
Disulfides,glutathionation	Cys	All, $ONOO^-$
Methionine sulfoxide	Met	All, $ONOO^-$
Carbonyls (aldehydes, ketones)	All esp (Lys, Arg, Pro, Thr	All
Oxo-histidine	His	γ-Ray, MCO
Dityrosine	Tyr	γ-Ray, MCO
Chlorotyrosine	Tyr	HOCl
Nitrotyrosine	Tyr	$ONOO^-$
Tryptophanyl modifications,	Trp	γ- Ray
Hydro (pero) xy derivatives	Val,Leu,Tyr,Trp	γ-Ray
Chloramines,deamination	Lys	HOCl
Lipid peroxidation adducts	Lys,His,Cys	γ-Ray, MCO
(MDA, acrolein, NHE)		
Amino acid oxidation adducts	Lys, His,Cys	HOCl
Glycosidation adducts	Lys	Glucose
Cross-links,aggregates,	Several	All

MCO; metal-catalyzed oxidation; All = γ-Ray, MCO, HOCl, ozone, O_2, MDA; Malondialdehyde.

1.2 Is oxidative modification reversible?

Reversible modifications, usually at Cys residues, may have a dual role of protection from irreversible damage and modulation of protein function (redox regulation). Irreversible modifications induced by ROS, such as di Tyr formation, protein-protein cross-linking, Lys

and Arg carbonylation are generally associated with permanent loss of function and may lead to either degradation of the damaged proteins (Berlett & Stadtman, 1997; Davies and Dean et al. 1997; Grune et al. 2003) or their progression into cytoplasmic inclusions, as observed in age-related neurodegenerative disorders (Giasson et al. 2000, 2002; Butterfield and Kanski, 2001).

1.3 Advantages of protein oxidation as a biomarker for oxidative stress

These include:

1. Relatively stable.
2. Types of modification reveal nature of the oxidizing agents (chlorotyrosine from HOCl, nitrotyrosine from ·NO and glutamic and aminoadipic semialdehydes from MCO).
3. Due to the specificity of protein functions, they have unique physiological consequences.
4. Availability of sensitive assays, which can detect as low as 1 pmol of oxidized product.

1.4 Radical-mediated protein oxidation

Protein oxidative modification is most likely to be mediated by free radical generation. *A free radical is any molecular species capable of independent existence that contains one or more unpaired electrons*. An unpaired electron is one that occupies an atomic or molecular orbital by itself. The presence of one or more unpaired electrons causes the species to be attracted slightly to a magnetic field (*paramagnetic*), and sometimes makes the species highly reactive (Cutteridge et al. 1989).The primary radical in most oxygenated biological systems is the superoxide ion ($O^{\cdot}_2$), which is in equilibrium with its protonated form, the hydroperoxyl radical ($HO_2^{\cdot}$). The major sources of these radicals are modest leakages from the electron transport

chains of mitochondria, chloroplasts, and endoplasmic reticulum. Although O^{-}_{2} is relatively unreactive in comparison with other radicals, biological systems can convert it into other more reactive species such as peroxyl (ROO·), alkoxyl (RO·) and hydroxyl radical (OH·).

1.4.1 Transition Metals

In their reduced forms, these metals catalyze the production of radicals from H_2O_2 via the Fenton reaction. From a radical point of view, the transition metals of interest are those that have variable valence which allows them to undergo changes in oxidation state involving one electron. This holds true for Fe, Cu and Mn but not Zn which has only one valence which, unlike Fe, Cu, and Mn, prevents it from promoting radical reactions.

1.4.2 Fenton Reaction

A mixture of H_2O_2 and Fe (II) salt reacts with many organic molecules, as was first observed by Fenton in 1894. The reactivity is most likely due to formation of the hydroxyl radical (·OH):

$$Fe^{+2} + H_2O_2 \leftrightarrows Fe^{+3} + \cdot OH + OH^{-}$$

Traces of Fe (III) can react further with H_2O_2:

$$Fe^{+3} + H_2O_2 \Rightarrow Fe^{+2} + O_2^{-} + 2H$$

Haber-Weiss reaction is a possible fate either iron- or copper-catalysed:

$$H_2O_2 + O^{-}_{2} \Rightarrow O_2 + OH^{-} + .OH$$

Further reactions are also possible:

$$O_2^{-} + Fe^{+3} \Rightarrow Fe^{+2} + O_2$$

$$\cdot OH + Fe^{+2} \Rightarrow Fe^{+3} + HO^{-}$$

1.5 Non-radical mediated protein oxidation

So far, two categories of non-radical intermediates in protein oxidation have been identified. Reactive moieties, notably DOPA, formed from tyrosine, can reduce transition-metal ions, thus enhancing reactions with hydroperoxides which are also able to induce radical

formation in reactions with O_2 (Davies, M. et al. 1997; Simpson J. et al. 1992; Gieseg, S. et al. 1993). The other category is hydroperoxides formed particularly on unsaturated aliphatic hydrocarbon side chains but probably also on main-chain α-chain carbons (Dean R. et al. 1996; 1995; Simpson, J. et al. 1992). These can be decomposed by transition-metal ions to give further radicals. The hydroperoxides can also be reductively detoxified to hydroxides probably without radical formation (Fu, S. et al. 1995).

1.6 An Overview for Oxidative Modifications of Different Amino Acids

1.6.1 Oxidative Modification of Protein Thiols

Thiol groups are easily oxidized by many ROS. Their modifications may have different physiological effects, depending on their reversible or irreversible nature. Mild oxidation of cysteines generates sulfenic acid, inter- or intra-molecular disulfides, protein mixed disulfides with low molecular weight thiols (e.g., GSH), and *S*-nitrosothiols. These reversible modifications can be part of regulatory processes of protein functions, in which cysteines can cycle between the oxidized and reduced state. Basically, sulfenic acid is unstable, being an intermediate in sulfinic and/or sulfonic acid generation. However, Cys residues can be irreversibly oxidized by strong oxidative agents to sulfinic and sulfonic acids, which cause loss of protein function and cannot be reversed by metabolic processes (Woo et al. 2003).

1.6.1.1 Possible Methods for Analysis of Oxidized Products of Protein Thiols

Formation of inter-molecular disulfides following oxidative insult generates macroscopic variation of protein molecular mass. However, the occurrence of mixed disulfides with low molecular weight compounds or intra-molecular disulfides, resulting in smaller changes in the molecular mass of intact proteins has been revealed by mass spectrometry. LC-ESI (light chromatography-electrospray ionization) or MALDI (Matrix-Assisted Laser Desorption/ Ionization) MS mapping experiments can detect mixed disulfides to specific Cys residues in

the polypeptide chain (Tell et al. 1998; Zheng Aslund and Storz, 1998; Vilardo et al. 2001; Cecconi et al. 2002). Irreversible oxidation of cysteine to sulfinic and sulfonic acids has been determined in intact protein molecules by using ESI-MS (Yang et al. 2002; Woo et al. 2003). These products are stable and insensitive to treatment with reducing agents. The identification of those irreversible products of modified cysteines was obtained on protein digests by MALDI or LC-ESI and confirmed by tandem MS analysis (Rabilloud et al.2002; Yang et al. 2002).

1.6.2 Oxidative Modification of Tyrosine

Specific tyrosine derivatives produced by oxidation have been characterized for: myeloperoxidase-catalysed reaction pathways through the action of HOCl (Cl-Tyr, and 3,5 -dichlorotyrosine, di-Cl-Tyr) (Domigan et al. 1995, Fu et al., 2000; Podrez, Abu-Soud and Hazen, 2000), eosinophil peroxidase-catalyzed reaction pathways through the action of hypobromous acid (3-bromotyrosine, Br-Tyr, and 3,5-dibromotyrosine, di-Br-Tyr) (Wu et al. 199), and free radical pathways (o-Tyr, *m*-Tyr, and *di*-Tyr). Many tyrosine modifications rely on nitration rather than oxidation reactions in which peroxynitrite is commonly implicated as the principal mediator of tyrosine nitration.

1.6.2.1 Possible Methods for Analysis of Oxidized Products of Tyrosine

Cl-Tyr, di-Cl- Tyr, Br-Tyr, di-Br-Tyr, NO_2–Tyr, and di-Tyr, have been all selected as amino acid products stable to acid hydrolysis, making them useful markers for protein oxidation/ nitration studies (Heinecke et al. 1999 b, c). On this basis, specific procedures for the detection of these derivatives in amino acid hydrolysates or biological fluids have been optimized by using direct HPLC quantification (Shigenaga et al. 1997) and GC (gas chromatography) or LC-MS analysis (Heinecke et al. 1999 b, c; Wu et al. 1999). However, methods for the detection of these modifications by direct ESI- or MALDI-MS

analysis of intact proteins or their peptide digests have found a positive application only in the case of NO_2-Tyr-, or di-Tyr-containing proteins and Tyr radical species (Minetti et al. 2000; Petersson et al. 2001; Sarver et al. 2001). The occurrence of intermolecular cross-linked di-Tyr residues in proteins following oxidative insult, to give macroscopic variation of protein molecular mass has usually been detected by low-resolution techniques such as SDS-PAGE under reducing conditions (MacMillan-Crow, Crow and Thompson, 1998; Lardinois, Medzihradszky and Ortiz de Montellano, 1999).

1.6.3 Oxidative Modification of Methionine

Met residues are highly susceptible to oxidation by almost all ROS (Vogt, 1995). Mild oxidizing conditions generate methionine sulfoxide, which can be further oxidized to methionine sulfone under stronger oxidizing conditions (Vogt, 1995; Levine, Moskovitz and Stadtman, 2000a). Methionine oxidation has been known to be associated with loss of protein function, as in case of alpha-1 antitrypsin, alpha-chymotrypsin actin & HIV-2 protease (Vogt, 1995; Davis et al. 2000; Dalle-Donne et al. 2002). However, Methionine oxidation does not cause loss of protein function in all cases (Levine et al. 1996). Interestingly, methionine oxidation can serve as an endogenous antioxidant defense, protecting the targeted proteins from more extensive irreversible oxidative damage at other essential amino acids (Levine et al. 1996; Levine, Moskovitz and Stadtman, 2000a) since it is enzymatically reversible. It has also been observed that, unlike other amino acid modifications, methionine modification has little or no effect on the susceptibility of proteins to proteolysis (Levine et al. 1996 and Stadtman et al. 2002).

1.6.3.1 Possible Methods for Analysis of Oxidized Products of Methionine

The most ideal and easiest method for the detection of the methionine oxidation products such as sulfoxide and sulfone is ESI or MALDI mass spectrometry of intact molecular species

or protein digests (Hanson et al. 1999, 2000). Identification of modified methionine residues has been obtained in different proteins by LC-ESI or MALDI mass mapping experiments (Hanson et al. 1999, 2000; Taggart et al. 2000). Localization of an oxidative modification to a specific Met residue has been carried out by database searching (e.g. with MASCOT or SEQUEST) or by *de novo sequencing (*PEAKS) with reference to the known amino acid sequence of the protein (Ahmed et al., unpublished).

1.6.4 Protein Carbonylation

Protein carbonylation is an irreversible oxidative modification. Protein carbonyls are not repaired and are either removed by proteolysis or accumulate as damaged or unfolded proteins (Stadtman and Berlett, 1998). Protein carbonylation is an important marker of protein oxidation and is reported to be a good indicator of the extent of oxidative damage of proteins associated with various human diseases (Levine et al. 2000; Dalle-Donne et al. 2003c). The most common human diseases associated with carbonylated proteins are ARDS, Alzheimer's disease, cataractogenesis, chronic renal failure, diabetes, pre-eclampsia, rheumatoid arthritis, ischemia, etc (Dalle-Donne et al. 2003). Carbonyl moieties (aldehydes and ketones) are produced on protein side chains, especially of proline, arginine, lysine and threonine, when these amino acids are oxidized into ketone or aldehyde derivatives (Berlett and Stadtman,1997). Protein carbonylation can also occur by reaction with various reactive products generated during lipid peroxidation, 4- hydroxynonenal (4-HNE), acrolein, and malondialdehyde (MDA). However, carbonyls can be induced *in vitro* by almost all types of oxidants, including site-specific metal catalyzed oxidation, HOCl, γ-irradiation and Ozone (Shacter, E. 2000).

1.6.4.1 Protein Carbonyls As Markers of Oxidative Stress

Protein carbonyls have a major advantage over lipid peroxidation products as markers of

oxidative stress in that oxidized proteins are more stable. Carbonyls are formed early and circulate in the blood for longer periods (at least 4 hours in serum) (Pantke, U. et al. 1999), compared with other parameters of oxidative stress, such as glutathione disulfide and MDA. Protein carbonyls are present at low levels in most protein preparations (~1 nmol /mg protein) (Shacter, E. 2000). *In vivo*, under conditions of oxidative stress, 2- to 8- folds elevations of carbonyls is expected (Shacter, E., 2000).

1.6.4.2 Possible Methods For Detection of Protein Carbonyls

Carbonylated adducts can be detected either in protein and fluid hydrosylates by GC- and LC-ESI-MS analysis or by direct ESI- or MALDI-MS measurements of intact proteins or peptide digests (Peterson et al. 1998; Saraswathi, Nakanishi & Shimizu, 1999; Lapolla, Fedele and Traldi, 2000). As well, protein modification by 4-HNE can be monitored for intact proteins by ESI- or MALDI-MS analysis (Bennaars-Eiden, A. et al. 2002, Crabb et al. 2002, Alderton et al. 2003). Furthermore, protein carbonyls can be measured within a mixture of proteins after reaction with dinitrophenylhydrazine (DNPH) by using spectroscopy, ELISA, immunohistochemistry or Western blot analysis (Shacter, E. 2000).

1.6.5 Oxidation Of Tryptophan

Tryptophan residues can be oxidized by ionizing radiation and are not expected to be oxidized by metal-catalyzed oxidation because tryptophan is not typically a site for metal iron binding (Stadtman, 1993). Several tryptophan oxidation products including kynurenine (KYN) hydroxytryptophans (HTRP), N-formylkynurenine (NFK), and 3-hydroxykynurenine (3OH-KYN) have been detected (Finley et al. 1998). Finely and co- workers (1998) have reported oxidative tryptophan modification in α-crystallin of bovine lens. Interestingly, the tryptophan oxidation products are themselves photosensitizers and are able to generate ROS (Reszka et al. 1996), which led to the hypothesis that oxidative tryptophan modification might play an

important role in senile cataractogenesis (Specter, 1995).

1.6.5.1 Possible methods for the detection of tryptophanyl oxidative modification products

Tryptophanyl oxidation products have been detected in protein digests by using MALDI-MS (Finely et al.1998). Taylor et al. (2003) have reported the use of LC-MS/MS and ESI-MS for the detection of tryptophanyl oxidative products.

1.6.6 Oxidation of Histidine

2-Oxo-histidine and 4- or 5-hydroxy-2-oxo-histidine are generated from histidine oxidation (Schöneich, 2000). Aspargine, aspartate and HNE-His can also be generated from histidine oxidation (Shacter, 2000). Histidine has been identified as the amino acid most susceptible to metal-catalyzed oxidation (MCO) *in vivo* e.g. Cu, Zn SOD, and *in vitro* e.g. Cu, Zn SOD, glutamine synthetase, glycated insulin and LDL (Schöneich, 2000). Histidine oxidation has been reported as one of the most important markers of oxidative stress *in vivo.*

1.6.6.1 Possible Methods For Detection of Oxidized Histidine products

The presence of oxidized histidine derivatives can be ascertained by direct ESI or MALDI-MS analysis of intact molecules (Kurahashi et al. 2001; Schöneich and Williams, 2002).

1.7 Study of Ferritin and Transferrin as a Model of Oxidative Modifications of Proteins

This section describes aspects of the chemical structure, physiological and biochemical functions of ferritin and transferrin.

1.7.1 Ferritin

Ferritin is the principal protein involved in storing iron in a soluble, non-toxic, yet bioavailable form in bacteria, plants, and animals. Ferritin maintains up to 4500 atoms of hydrolyzed and polymerized atoms in a soluble form within a protein shell with a hollow interior of approximately 90 Å (Hynes, M. et al. 2002; Welch, K. at el. 2002).

Ferritin binds iron in a catalytically inactive manner; accordingly, oxidative reactions cannot be promoted by iron bound to ferritin. Because the main iron pool of most cells is located within the ferritin core, it is important to know under what circumstances this pool may become a source of free iron capable of causing oxidative damage (Rudeck, M. et al. 2000). Ferritin iron can be released by a variety of reducing agents [dihydroflavins, paraquat, iron chelators and, interestingly, superoxide ion] (Thomas Paul, 2000), due to their ability to reduce tightly bound iron (III) to the more mobile iron (II) form (Boyer et al. 1987; Thomas et al. 1986). Direct chelation of iron (III) may also mobilize iron from ferritin with or without reduction to iron (II). In the absence of chelators, the reduced core of ferritin iron appears to remain within the protein shell (Hynes et al. 2002). However, ferritin has been shown to release iron on contact with activated microglia and neutrophils by a superoxide-dependent mechanism (Rudeck, M. et al. 2000). Once oxidative damage occurs to ferritin under the effect of any of the above-mentioned circumstances, the ferritin molecule undergoes some physiological malfunctions such as release of iron and possible damage of the apoferritin

molecule (Rudeck, M. et al. 2000). Hydrogen peroxide-induced protein oxidation enhances the proteolytic susceptibility of the damaged ferritin (Davies, K. et al. 1987; Reinheckel, T. et al. 1998; Grune, T. et al. 1995). Mammalian ferritin consists of a protein shell with 24 subunits surrounding a core of ferrihydrate, each subunit weighing either 19 766 kDa (the L subunit) or 21 099 kDa (the H subunit) (Hynes et al. 2002; San-Marina, S. et al. 1995 and Theil, 1987). In normal human subjects, 25% of the total body iron is found in ferritin. Brain ferritin has 60-70% H subunit content, while horse spleen ferritin and mammalian liver ferritin consists predominantly of L subunits. Brain ferritin has been reported to have iron saturation of 1500 iron atoms per ferritin molecule (Hynes et al. 2002; Fleming et al. 1987; Dedman et al. 1992). Ferritin may play an important role in ameliorating metal toxicity when the brain is exposed to high levels of toxic metals. The increase in brain iron associated with several neurodegenerative diseases may lead to an increased production of free radicals, mostly of the hydroxyl radical (· OH) via the Fenton reaction. However, depending on the availability of free iron, the Fenton reaction can be directed as shown in the following formula:

O^{-}_2 (superoxide ion)- - - ----$\xrightarrow{SOD}$ H_2O_2

H_2O_2------ ----------$\xrightarrow{\text{Catalase \& Reduced Glutathione}}$ H_2O OR ·OH (whenever free iron is available).

As shown, O^{-}_2, under the action of superoxide dismutase (SOD) is converted into H_2O_2 (hydrogen peroxide), which may be converted into water in the presence of catalase and reduced glutathione or into the fatal hydroxyl radical (·OH) whenever free iron is available. It is believed that iron loads into ferritin spontaneously, dependent upon a “ferroxidase activity “of the H subunits of ferritin (Welch et al. 2002). Loading iron into ferritin via its ferroxidase activity could damage the ferritin and any other biomolecules in close proximity as reported by Welch and co-workers, 2002.

According to the method of Silva, D. et al. (1992), the loading of apoferritin with ferrous ammonium sulfate was dependent on buffer, pH and the presence or absence of chelator, and was directly related to the rate of iron autoxidation. These authors indicated that iron loading into ferritin core resulted in depletion of the basic amino acids lysine and histidine, probably as a result of protein oxidation, as well as release of more iron than other methods for iron incorporation. Silva, D. et al. (1992) reported that buffers such as, MOPS, or the Good's buffers, which are poor chelators of iron, allow rapid autoxidation of Fe (II) at pH 7.0. They have proposed that ferrous iron forms a complex with apoferritin prior to oxidation and incorporation, since ferric iron presented to the apoferritin under identical conditions does not result in incorporation. HEPES buffer, which is also a poor chelator of iron, allows the greatest oxidation of ferrous iron since it does not compete well, either with the protein for metal binding, or with water for coordination to free sites on the metal. When ferrous ammonium sulfate was incubated with ferritin in HEPES buffer at pH 7.0, the initial rate of iron incorporation was approximately 10-fold greater than when the experiment was repeated at pH 6.0. It should be noted that, as the pH decreased, the stability of Fe (II) increased which may hinder the hydrolysis and autoxidation because of the lack of the deprotonation of iron-bound water molecules (Silva, D. et al. 1992).

1.7.2 Transferrin

The transferrin family (Trf) constitutes the major iron transport and /or scavenging system in vertebrates and some invertebrates (Aisen, 1998). However, Trf can also function as an iron chelator, which contributes to host defense by limiting iron availability for microbial pathogens. This capacity to bind iron also enables transferrin to protect cells from oxidative damage. ·OH radical generated from $^{-}O_2$ and H_2O_2 via the Haber-Weiss reaction has been suggested to be an important contributor to phagocyte- mediated tissue injury and other free

radical associated processes. Trf is a soluble glycoprotein and a bilobal molecule i.e. it contains an N-terminal (amino acids 1-336) and a C- terminal (amino acids 337-679) globular domain. Each domain contains a metal-binding site and each lobe binds one iron atom. Under normal conditions, these two high-affinity-iron binding sites, prevent the existence of measurable amounts of unbound iron in the plasma. The main source of iron for transferrin is catabolism of non-viable red blood cells and its main destination is the erythroid marrow. Amino acid sequences of transferrins from several species, including human, bovine, rabbit, and chicken, show a high degree of sequence similarity (Thevis, M. et al. 2003). Serum-transferrins and ovotransferrins transport iron from neutral biological fluids to the cytoplasm by receptor-mediated endocytosis (El-Hage Chahine, et al. 1999; Dautry Varsat et al. 1983). In contrast, lactoferrins and again ovotransferrins behave as bacteriostatic iron scavengers (Moore et al. 1997; Kurokawa et al. 1995). As we have mentioned earlier, free iron is capable of stimulating the production of free radicals which cause oxidative damage such as lipid peroxidation. Trf is one of the most important antioxidants, and acts by sequestration of iron in a redox-inactive form (van Campenhout, A. et al. 2003). Apotransferrin, at physiological concentrations (2-3 mg/ml) is known to inhibit lipid peroxidation in the liposome model by iron-binding (Van Campenhout et al. 2003). However, at higher concentrations, apotransferrin does not inhibit lipid peroxidation any further, while at low concentrations, apotransferrin reduces antioxidant capacity in a concentration-dependent manner. This finding is clinically relevant in diseases that are associated with lower plasma transferrin concentrations as a result of either a decreased synthesis or increased breakdown (Van Ca penhout et al.2003). Trf is a negative acute-phase protein which is downregulated in inflammatory diseases such as diabetes. Moreover, oxidative damage results in protein fragmentation and nephropathy which can lead to loss of transferrin in the urine of type 1 and

type 2 diabetic patients. Interestingly, microtransferrinuria seems to be a more sensitive index for renal dysfunction than microalbuminuria (Campenhout,et al. 2003). Keenoy et al. (2005) have also reported that inhibition of lipid peroxidation by transferrin was almost total in the presence of 60 μM iron, a concentration that saturates transferrin completely (100% transferrin saturation).

1.7.2.1 NTBI (Non-transferrin bound iron)

Plasma transferrin has considerable reserves for coping with increasing amounts of incoming iron, but these may be exceeded in certain pathologic diseases. In acute iron poisoning, the concentration of NTBI ranging from 128 μM to over 800 μM have been documented, exceeding the total iron binding capacity of transferrin (Reynolds and Klein, 1985). Buchanan (1971) has reported acute episodes of abdominal pain and shock in association with extremely high serum iron measurements ranging from 214 μM to 2200 μM in severe idiopathic hemochromatosis patients. NTBI is weakly complexed to albumin, citrate, amino acids and sugars , and behaves differently from iron associated with transferrin. Lehotay, D. et al. (2001) have indicated that patients with HH (hereditary hemochromatosis), but not other groups with iron overload status, exhibit higher than normal levels of NTBI as compared to control groups without hemochromatosis.

1.8 Hydrogen Peroxide (H_2O_2) As an Oxidizing Agent in Biological Systems

Hydrogen peroxide is generated in all aerobic organisms as a result of normal cellular metabolism. Hydrogen peroxide is known to be produced during photosynthesis, and during phagocytosis as well; it can also be detected in human expired air. It can penetrate cell membranes rapidly whereas superoxide ion usually cannot. Once inside the cell, it can react with Fe (II) or Cu (I) via Fenton reaction. Hydrogen peroxide is being continuously generated and destroyed. The liver has the most effective mechanism for disposing of hydrogen peroxide. Other tissues such as lens of the human eye and rabbit spermatozoa have less effective hydrogen peroxide-removal mechanisms that causes release of it into the surrounding media (Cutteridge et al. 1985). Thus, electrons that leak from the electron transport chain of mitochondria cause the univalent reduction of molecular oxygen to superoxide anion ($O_2^{\cdot -}$), which is then spontaneously or enzymatically dismutated to H_2O_2 (Kim et al. 2000). Hydrogen peroxide is also generated by lipoxygenase, xanthine oxidase, and cytochrome P450, as well as in the cellular response to ultraviolet radiation (Kim et al., 2000). There are also several enzymes that produce hydrogen peroxide without intermediacy of free O^{-}_2 radical. These include glycollate oxidase, D-aminoacid oxidase, and urate oxidase (Cutteridge et al. 1985). Furthermore, many cell types produce hydrogen peroxide in response to a variety of extracellular stimuli including cytokines, peptide growth factors, neurotransmitters, hormones and (Kim, et al. 2000).

Hydrogen peroxide is readily converted to hydroxyl radical ($^{\cdot}OH$), as mentioned above, via the Fenton reaction in the presence of iron (or copper) and cellular reductants, and these radicals induce irreversible oxidative damage to various cellular components (Kim et al. 2000).Two enzymes are known to be involved in protecting living cells against hydrogen

peroxide:catalase and peroxidase. Catalase catalyzes the breakdown of hydrogen peroxide into water and oxygen. Catalase activity can be inhibited by azide or cyanide and importantly, aminotriazole, which when fed to rabbits inhibits catalase activity causing the concentration of hydrogen peroxide to increase leading to an accumulation of hydrogen peroxide in the lens, inducing cataractogenesis. Glutathione peroxidase cannot by itself prevent the hydrogen peroxide generation (Cutteridge, et al. 1985). Glutathione peroxidase exerts its action as scavenger of hydrogen peroxide by catalyzing the oxidation of GSH to GSSG (Cutteridge et al. 1985). Since the O-O bond is relatively weak, hydrogen peroxide decomposes easily by homolytic fission to give rise to the hydroxyl radical. It mixes readily with water, and its oxidizing action gives it some antibacterial properties when used in high concentrations (Cutteridge et al. 1985).

1.9 Mass Spectrometry As an Analytical Technology

Mass spectrometry has become an important technique to study protein sequence and structure. This has been achieved, in part, by improvements in ionization and mass analysis techniques concurrently with large-scale DNA sequencing of whole genomes (Yates, 1998). Mass spectrometry can measure the masses of atoms or molecules that have been converted into ions. As shown in Fig.1, a generic mass spectrometer consists of:

- **Inlet:** through which the sample (solid, liquid or gas) is introduced into a vacuum chamber.
- **Ion Source:** in which the sample is ionized.
- **Mass Analyzer:** in which ions are separated according to their mass-to-charge *(m/z)* ratios.
- **Detector:** in which the ions generate an electrical signal that is proportional to the number of ions.
- **Data Analysis System:** which records these electrical signals and then converts them into a mass spectrum (a plot of relative ion abundance vs. m/z ratio). A good mass spectrum should contain sharp, symmetrical, and well defined peaks and have an acceptable resolution and signal-to-noise ratio.

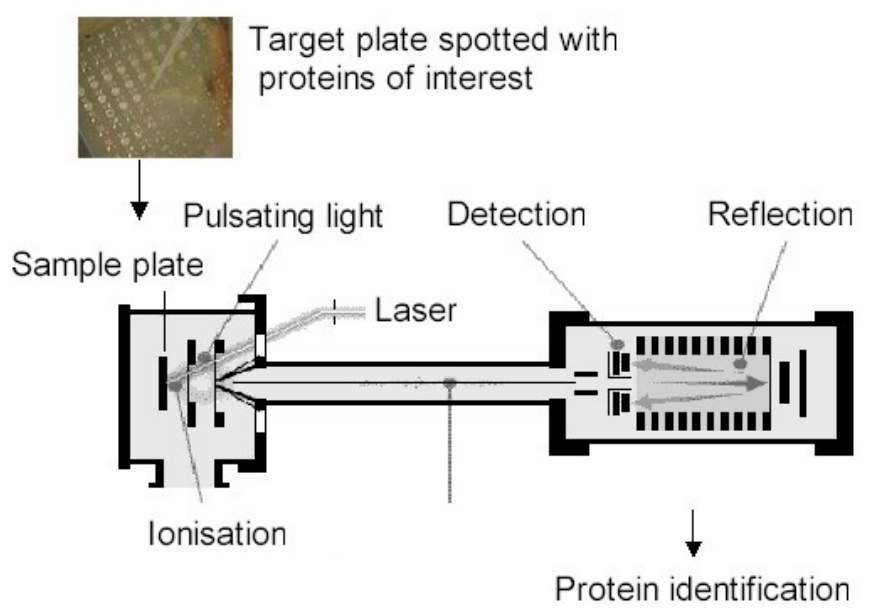


Fig.1: Schematic diagram of a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems)

1.9.1 Key Technologies In The Study of Proteins

1.9.1.1 One-Dimensional Polyacrylamide Gel Electrophoresis (1-D PAGE)

The separation of proteins at this stage is based on differences in molecular weight.

1.9.1.1 Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS)

This technique provides rapid identification of PAGE or affinity-purified proteins by mass analysis and database matching of component peptides. MALDI-MS is normally used to analyze relatively simple peptide mixtures. Peptide mass mapping can positively identify a protein of known sequence if peptide masses match to within 50 PPM and if the protein sequence coverage is at least 15%. MALDI uses a pulsed UV to desorb and ionize compounds that have been co-crystallized in a UV-absorbing matrix (Fig.2).

MALDI works with a time-of-flight (TOF) analyzer that measures the masses of intact peptides. TOF analyzers have 2 modes of operation; Linear, which is more sensitive with lower mass resolution; and Reflectron, which is less sensitive with higher mass resolution. When acquiring data, there are some factors that contribute to the accuracy of mass measurements:

1. Symmetrical peak shape: Depending on the *m/z* in question, the spacing between data points can represent a change in mass from 10 to 20 ppm to more than 100 ppm.
2. Signal-to-noise ratio as high as possible without skewing peak shape: peak position can be affected by noise or by saturation of the detector.
3. Signal intensity of reference and analyte peaks above *m/z* 1,000: Needed for accurate assignment of peak position.
4. Signal intensity of reference and analyte peaks should be in the same range.
5. No contaminants should be present.

6. Two internal reference masses that bracket the mass range of interest.

Most instruments (e.g. Voyager-DE STR, Applied Biosystems) have software that includes a default calibration routine that provides adequate mass accuracy for many applications. The calibration function in the Voyager (Data Explorer) software allows the generation of four types of calibrations:

1. Default calibration: provides typically 0.1% accuracy in Linear Mode, and typically 0.01% or better accuracy in Reflector Mode.
2. External calibration: Provides 0.05% accuracy in Linear mode, and 0.01% or better accuracy in Reflector Mode.
3. Internal calibration: Provides accuracy of 0.02% or better in Linear Mode, and 0.002% (20 ppm) in Reflector Mode.
4. Sequence control calibration: Provides external, internal, and internal with automatic updating calibration options during acquisition from the Sequence Control Panel.

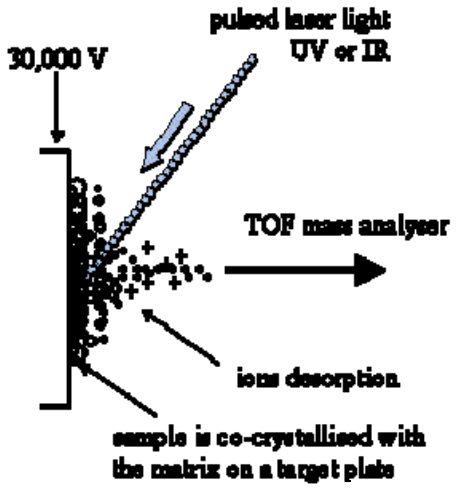


Fig.2: Mechanism of desorption and ionization during MALDI

1.9.1.2 Electrospray Ionization Tandem Mass Spectrometry (ESI-MS/MS)

ESI-MS/MS allows proteins and posttranslational modifications (PTMs) to be identified by sequence analysis of component peptides. ESI-MS/MS systems are preferred for the analysis of complex samples. ESI-MS/MS sequencing is also used when peptides cannot be matched to proteins in existing databases (Wilm et al. 1996). ESI-MS/MS can be performed using ion trap and tandem quadrupole instruments, and is used to generate fragmentation spectra (CID spectra) of selected precursor ions. In this study, our samples were analyzed by LC-MS/MS using a capLC ternary HPLC system (Waters, Milford, MA, USA) interfaced to a Q-TOF Ultima Global hybrid tandem mass spectrometer fitted with a Z-spray nanoelectrospray ion source (Micromass, Manchester, UK). To maximize the formation of peptide ions by ESI, separations are best conducted at low pH (Ross, A. 1998). The purpose of using tandem mass spectrometer (MS/MS) is to generate fragment ions from parent ions to provide structural information about a given molecule, as used in our *in vitro* and *in vivo* ferritin and transferrin oxidation experiments. Tandem mass spectrometry also allows for identification of compounds in complex mixtures. This is also demonstrated in our *in vivo* experiments which dealt with plasma samples from patients with hereditary hemochromatosis, since our protein of interest (transferrin) was identified among several plasma proteins. Different MS/MS configurations include; Quadrupole- quadrupole (low energy),Magnetic sector-Quadrupole (high energy), Q-TOF (low energy) and TOF-TOF (high energy).

1.9.1.3.1 MS/MS for protein ID

Proteins of interest are isolated from gel and subjected to tryptic digestion. Trypsin cleaves at the C-terminal side of lysine and arginine. Peptides are sent through the ionizer and into a collision cell where *singly or multiply charged i*ons are selected and fragmented through

collision induced dissociation (CID). The resulting fragment ions are analyzed to determine the sequence or to identify the parent peptide. CID of peptides less than 2-3 KD is most efficient for MS/MS studies. The frequency of tryptic cleavage sites guarantees that most peptides are of this size. Protein ID using peptide CID spectra is more specific than that achieved by mass mapping because, in addition to the peptide mass, the peak pattern in the CID spectrum also provides information about peptide sequence.

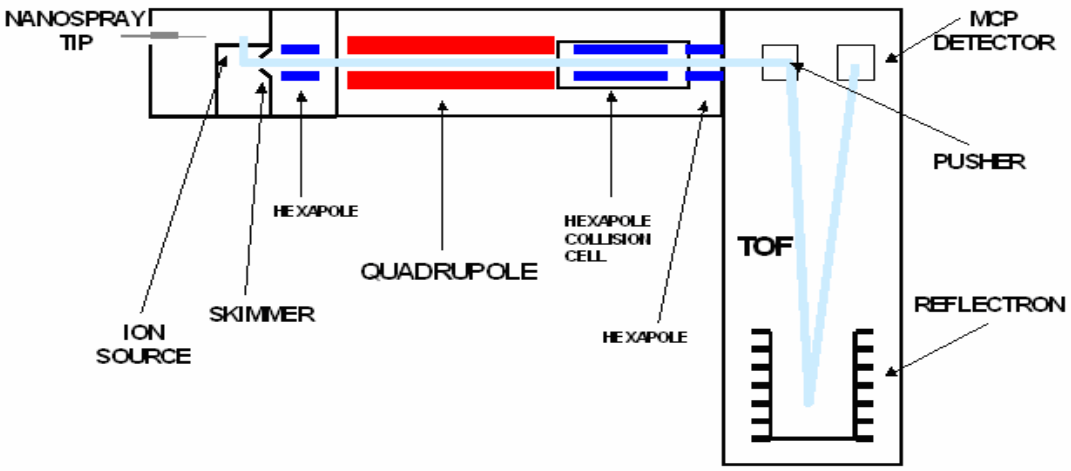


Fig. 3: Schematic diagram of Quadrupole-Time-OF-Flight (Q-TOF) Tandem Mass Spectrometer

1.9.1.3.2 Peptide fragments and daughter ions

Peptides, once admitted to the collision cell, usually fragment at the weakest bond (the peptide bond) although some CH-NH and CH-CO cleavage also occurs. Peptides do not fragment sequentially, that is to say, the first fragmentation event does not start at the amino terminus and proceed sequentially one residue at a time down the amino acid chain. The fragmentation events are somewhat random and not necessarily sequential. In addition, some fragmentations are preferred over others. Upon fragmentation, two main types of daughter ions are produced; **b**- and **y**- ions. These are the most common types of daughter ions.

During CID, peptides are preferentially fragmented along the peptide backbone. Six types of fragment ions that may be generated. These include **a**, **b** and **c,** if the charge is located at the N-terminus and to fragments of type **x**, **y** and **z** if the charge is located on the C-terminus. Any position in the peptide backbone can be fragmented including alkyl-carbonyl bonds (CHR-CO), producing **a** and **x** ions, peptide-amide bonds (CO-NH), producing **b** and **y** ions and amino-alkyl bonds (NH-CHR), producing **c** and **z** ions. Cleavage of amide bonds is most common generating **b**- and **y**-type ions. Additional fragment ions resulting from the loss of water, carbon monoxide or ammonia can also be generated during CID and facilitate the identification of specific amino acid within a spectrum (Schweppe et al., 2003).

1.9.1.3.3 MS/MS sequencing and calculation of b- and y- ions masses

It is not always easy to differentiate between **b**- and **y**-ion. Because trypsin cuts after lysine or arginine, we can assume that **y**$_1$ corresponds to one of these two residues. The *m/z* of **y**$_1$ is calculated by adding 19.018 u (three hydrogens and one oxygen) to the residue masses of lysine or arginine. **y**$_2$ can be calculated using the same method by finding another prominent peak that is equal to **y**$_1$ + AA, where AA is one of the known residue masses for each of the

20 common amino acids, and so on for each $\mathbf{y}_n$. Hence, the $\mathbf{y}_n$ series produces a reverse sequence of the peptide. **b**-ions can be constructed using the same strategy. The fragment containing only the amino terminal amino acid is termed $\mathbf{b}_1$. The $\mathbf{b}_1$ fragment is seldom observed making it difficult to determine the order of the first two N-terminal amino acids in a peptide sequence. The fragment containing the first two amino terminal amino acids is termed the $\mathbf{b}_2$ ion, and so forth. Once the mass of a **b** or **y** ion is determined, the corresponding **y** or **b** ion can be calculated using the following formulas:

$\mathbf{y} = (M+H)^{1+} - \mathbf{b} + 1$

$\mathbf{b} = (M+H)^{1+} - \mathbf{y} + 1$

1.9.1.3.4 Different MS/MS instruments yield different spectra

A typical Q-TOF or Triple quad MS/MS spectrum of a tryptic peptide contains a continuous series of **y-**type ions. The **b**-type ions are usually seen only at lower masses below the precursor m/z value. Ion trap CID data of tryptic peptides contains a continuous series of both **b**-type and **y**-type ions throughout the spectra.

1.9.1.4 High Performance Liquid Chromatography (HPLC)

Increases sensitivity and sequence coverage for ESI-MS/MS by providing on-line separation of peptide mixtures.

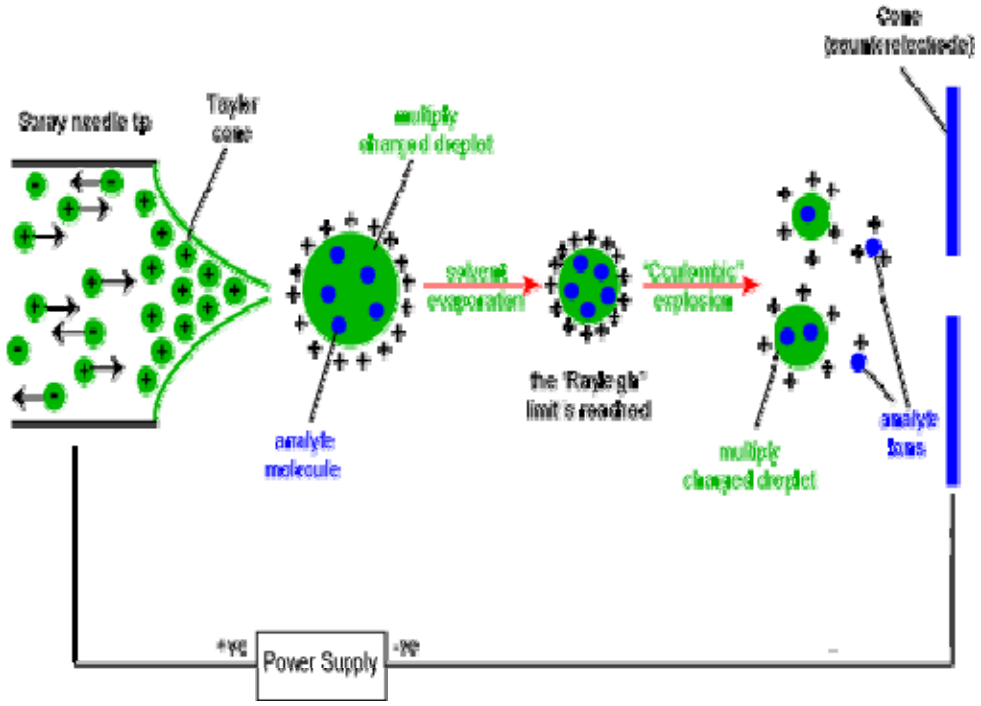


Fig: 4: The formation of the charged droplets by Electrospray.

1.9.1.5 PEAKS Software

This software can be used to interpret peptide MS/MS data directly and/or correlate it with sequences in databases. The rapid increase in the number of protein sequences in these databases have greatly aided the analysis of tandem mass spectra of peptides, since deducing peptide sequences from raw MS/MS data is tedious and slow when performed manually. The most popular software used to search databases of known genomes with the uninterpreted MS/MS data are Mascot and Sequest. These algorithms are effective but often give false positives or incorrect identification (Ma, B. et al. 2003).

Searching database with partial sequences (sequence tags) derived from MS/MS data gives more reliable results (Mann, M. et al. 1994). The *de novo* sequencing software package, PEAKS, has the advantage of extracting amino acid sequence information without the use of databases. PEAKS uses a new model and a new algorithm to efficiently compute the best peptide sequences whose fragment ions can best interpret the peaks in the MS/MS spectrum.

The output of the software gives amino acid sequences with common and user-defind posttranslational modifications, as well as an additional novel positional scoring scheme for entire and partial sequences (Ma, B. 2003).

PEAKS has been used to successfully compute the sequences of peptides, and is a very useful tool for the analysis of proteomes of both known and unknown genomes.

1.10 Hereditary Hemochromatosis

Iron overload is a common problem found in clinical practice. Iron overload can be either primary (as a result of an inherited metabolic disorder) or secondary to another problem that results in iron absorption (Harrison S. et al. 2003). The most common cause of primary iron overload is hereditary hemochromatosis (HH). However, among secondary iron overload causes are iron-loading anemias e.g. Thalassemia major, Sideroblastic anemia, chronic hemolytic anemia and aplastic anemia. Moreover, red blood cell transfusions, iron-dextran injections, long term hemodialysis, porphyria cutanea tarda, hepatitis C and B non-alcoholic steatohepatitis, aceruplasminemia and congenial atransferrinemia are possible causes for secondary iron overload (Harrison, S. et al. 2003).

1.10.1 History

The term 'hemochromatosis' was first used by von Recklinghausen, a German pathologist in the late 1800s (Recklinghausen, V. et al. 1889); he determined that the pigmentation seen in patients with advanced hemochromatosis was due to iron. In 1935, Joseph Sheldon, a British geriatrician, published a monograph describing over 300 cases with hemochromatosis, concluding that the disorder was an inherited defect caused by excess iron (Sheldon, J. 1935).

In mid-1970s, Simon and colleagues in northern France confirmed that hereditary hemochromatosis (HH) was inherited as an autosomal recessive disorder linked to the short arm of chromosome 6 in the region of HLA-A3 (Simon, M. et al. 1977). Finally, in 1996, a team of molecular geneticists in California discovered the gene responsible for HH (Feder, J. et al. 1996). It was first called *HLA-H* and was later named *HFE* (Harrison, S. et al. 2003).

1.10.2 HFE gene and protein

As mentioned above, Feder and colleagues used a positional cloning technique to identify a

novel gene called *HFE* in the HLA region of chromosome 6 (Feder, et al. 1996 and Harrison et al. 2003). This gene encodes a MHC class 1-like protein that binds to β2-microglobulin (β2M) and that interacts with TfR-1 (Enns, et al. 2001 and Lebron, et al. 1998). Uncomplexed HFE protein is rapidly degraded (Whittington, et al. 2002) HFE and transferrin competes for overlapping binding sites on TfR (Proceeding of BioIron, 2002). Interaction between HFE and TfR is probably necessary in the normal control of iron absorption, as compound mutant mice hemizygous for TfR and HFE -/- accumulate more liver iron than HFE -/- mice alone (Harrison, et al. 2003 and Levy, et al. 1999). HFE protein has a large extracellular domain, a single transmembrane region, and a short cytoplasmic tail. Two missense mutations were identified in *HFE* (Feder, et al. 1996 and Harrison et al. 2003). The most common one (C282Y) results in the change of a cysteine to a tyrosine at amino acid position 282. This amino acid substitution prevents the binding of HFE protein to β2M by disrupting a disulfide bridge in the extracellular domain and thereby decreases the amount of HFE protein expressed on the cell surface (Enns et al. 2001; Britton et al. 2002; Trinder et al. 2002, and Lebron et al. 1998). Failure to interact with β2M impairs HFE protein processing, transport, folding and cell surface expression (Waheed et al. 1997). Numerous studies have confirmed that approximately 85% of typical HH patients from around the world are homozygous for C282Y (Bacon et al. 1999 and Adams et al. 2000). In the original work conducted by Feder and colleagues, 83% of 178 phenotypic HH patients were found to be homozygous for the C282Y mutation Feder et al. 1996 and Harrison et al. 2003). The second mutation in *HFE* results in a change in histidine to aspartate at amino acid position 63 (H63D) and is found in 15-20% of the general population (Bacon et al. 1999 and Adams et al. 2000).This mutation doesn't inhibit the binding of HFE protein to β2M, and individuals who are homozygous for the H63D mutation have only mild iron accumulation (Gochee et al. 2002).

However, some individuals who carry both the C282Y and H63D mutations (compound heterozygotes) have clinically significant iron overload (Harrison et al. 2003; Bacon et al. 1999 and Adams et al.2000). *HFE* knockout mice studies validated the importance of *HFE* in the development of iron overload, providing evidence that these *HFE* knockout mice have increased iron absorption, elevated transferrin saturation levels and increased hepatocellular iron storage similar to humans with *HFE*- linked HH (Zhou et al. 1998 and Bahram et al. 1994).

1.10.3 Pathophysiology of HH

The primary defect in *HFE*-related HH is an increase in intestinal iron absorption relative to body iron stores (Harrison et al. 2003). Normally, total body iron content ranges from 3-5 g, and 20-30 mg of iron are recycled daily, mainly through phagocytosis of senescent erythrocytes by macrophages (Andrews, 1999). Only 1-2 mg of iron /day are typically lost from the body, and females have an increased physiologic loss of iron through menstruation. Accordingly, 1-2 mg of iron are absorbed daily from the diet to maintain normal iron homeostasis (Andrews, 1999). The cells of the intestinal epithelium in the duodenum and proximal jejunum are responsible for absorbing iron from the diet (Fletcher et al. 2002). Iron enters crypt cells of the duodenum from the circulation by transferrin-mediated endocytosis involving HFE protein, iron-laden transferrin and TfR (transferrin receptor). Crypt cells sense body iron requirements and are programmed with this information as they mature into villous cells (mature enterocytes), which absorb dietary iron. Non-heme ferric iron (Fe III) is reduced to ferrous iron (Fe II) by Dctyb protein and is transported into the cells by DMT1. Iron may be either stored in the cytoplasm of the villous as ferritin, or exported into the circulation laden by transferrin through Ireg1 with the aid of hephaestin (Heph) which functions as a ferroxidase, oxidizing Fe II into Fe III. Transferrin transports Fe III in the

circulation to sites of utilization, particularly the erythrocyte precursors in the bone marrow. Macrophages of the reticuloendothelial system take up iron via transferrin-receptor mediated endocytosis and recycle iron from senescent red cells back into circulation. In the liver, hepatocytes acquire iron from various sources including receptor-mediated endocytosis via TfR1 and the TfR2. DMT1 may be involved in the entry of nontransferrin bound iron (NTBI) into hepatocytes. Within the endosome, acidification results in iron release from the receptor Iron may be stored as ferritin until required. The liver is the main storage site for iron as ferritin (Fletcher et al. 2002). The released iron is once again accepted by transferrin to be transported in the circulation. Based on the previously mentioned information, iron homeostasis is regulated under the umbrella of two regulators (sensors), **'**erythroid regulator' and 'iron stores regulator' (Fletcher et al., 2002). The 'erythroid regulator' has much greater control over iron absorption because of the high demand for iron from these cells, which must be met on a daily basis. The regulatory signal is sent to the liver and other storage sites to stimulate the rapid mobilization of iron for erythropoiesis. Following the depletion of iron stores from storage sites, the proposed 'stores regulator' stimulates pathways responsible for the slow absorption of iron from the diet. This signal has to be controlled to prevent excessive absorption (Sayers et al., 1994). Hepcidin is another component of the 'store regulator' expressed in the liver and induced by iron. It acts to down regulate iron absorption (Andrews, 1999 and Harrison et al. 2003).

1.10.4 Diagnostic criteria and treatment

The first step in the definitive diagnosis of HH is the use of phenotypic tests of iron overload. The 'gold standard' is transferrin saturation (serum iron divided by transferrin) (Fletcher et al. 2002). Some other markers are also used e.g. UIBC (unsaturated iron binding capacity), TIBC (total iron binding capacity) (Sayers et al. 1994). Serum ferritin and liver enzymes

(particularly ALT) are also useful when the patient is being considered for liver biopsy. So far, two categories of patients can be identified, asymptomatic and symptomatic patients (Fletcher et al. 2002). Asymptomatic patients present with abnormal serum iron whilst symptomatic patients usually present with fatigue, diabetes mellitus, skin pigmentation, hepatomegally, hypogonadism, arrhythmia and arthopathy (Tavil, 2001). However, patients may also be evaluated following family or population screening using phenotypic and/or genotypic methods (Tavil, 2001). Genetic studies usually involve analysis for both the C282Y mutation and H63D in *HFE*. If the subject is C282Y homozygous, with elevated transferrin saturation and/or serum ferritin and/or elevated liver function tests, liver biopsy should be considered. Furthermore, if the serum ferritin exceeds 300 μg L^{-1} in males or 200μg L^{-1} in females and continues to rise, venesection therapy should be started (Fletcher et al. 2002). Regular venesections of 500 mL (250 mg iron)/treatment should be continued until the hemoglobin begins to fall and does not rise again immediately after venescetion ceases. Serum ferritin should fall below 50 μg L^{-1}. If the subject is C282Y homozygous with normal transferrin saturation, serum ferritin should be measured annually and venesection therapy considered if these markers become elevated. For those subjects who are heterozygous for the C282Y mutation or compound heterozygous (heterozygous for both C282Y and H63D mutations), liver biopsy should be considered using the same criteria. Moreover, some other co-factors should be considered e.g. steatohepatitis, excess alcohol consumption or chronic viral hepatitis. Those with normal iron should be counseled. Similar considerations should be given to those subjects who are C282Y normal or homozygous for the H63D mutation (Fletcher et al. 2002).

2.0 Hypothesis

We hypothesized that disrupting iron homeostasis plays a role in free radical injury and leads to oxidative modifications and possible loss of function of ferritin and transferrin in hereditary hemochromatosis (HH) when free iron or non-transferrin-bound iron (NTBI) concentrations rise and iron-binding proteins become saturated.

3.0 Rationale

This study was conducted to shed light on the role of oxidative modifications of ferritin and transferrin and to determine the nature of such oxidative damage in hereditary hemochromatosis (HH). Iron overload in HH may lead to oxidation of these proteins that could cause a loss of function.

Such information will provide insight into the structure–function relationship of these proteins, and may improve our understanding of the altered role of these oxidatively modified proteins.

4.0 Objective of the Study

- Validation of the methods.
- Comparative analysis of ferritin and transferrin in plasma from normal individuals and HH patients.

5.0 Materials and Methods

5.1 'Gel-free' Protein Digest Experiment

The purpose of this experiment was to demonstrate 'gel free" protein digestion while confirming the identities of commercial standard proteins (ferritin and transferrin) by peptide mass fingerprinting using MALDI-TOF/MS. Proteins were analyzed without exposure to oxidative stress. This experiment had two stages: **Poroszyme Method for Protein Digestion** and **MALDI-TOF MS** to identify the proteins by peptide mass fingerprinting. We used 60 ng of the ferritin standard (150 femtomole of protein), and 6 ug of the transferrin standard (~ 78 picomole). These quantities represent, respectively, 100 times and 0.006 times the minimum amounts of ferritin and transferrin normally found in 50uL of human plasma (the volume of sample used later for immunoprecipitation of these proteins from human plasma samples). Page 45.

5.1.1 Poroszyme™ Method for Protein Digestion

1. Approximately 100 µL of sample was dried down on a speed vac at 35º C for ~ 1½ hrs.
2. The dried sample was dissolved in 100 µL using 100 mM ammonium bicarbonate buffer containing 60 mM dithiothreitol as a reducing agent.
3. The mixture was heated for 1 hr at 45º C.
4. Iodoacetamide was added in ammonium bicarbonate buffer to a final concentration of 190 mM.
5. The samples were heated for 1 hr at 37ºC in dark.
6. 5 µL of Porozsyme beads (immobilized trypsin) were added to each sample and vortexed for 1 hr.

7. The samples were centrifuged to pellet beads for 10 min at 10,000 g.
8. The supernatant was transferred to a new tube; without disturbing the beads.
9. 10 μL were transferred to a smaller tube for solid-phase extraction using Millipore Zip Tips
10. 10 μL 1% TFA in water was added to each.
11. Using Millipore Zip Tip* - C_{18} reversed phase (size P10) and a 10 μL pipette:
    - Aspirate with 75% ACN with 0.1% TFA. Dispense to waste 3 x 10 μL
    - Aspirate with 0.1% TFA in water. Dispense to waste 3 x 10 μL.
    - Samples were aspirated and dispensed in tubes 5 to 10 times. Tips were blown out.
    - Tip was washed with 75% ACN with 0.1% TFA. Aspirate and dispense to waste 5 x 10 μL.
    - Aspirate with α C matrix (5 mg/ml in 75% ACN with 0.1% TFA) to first indent on tip.
    - Dispense onto plate target spot and mix, avoid forming bubbles on plate.

*ZipTip is a 10 µL pipette tip with a 0.6 or 0.2 µL bed of chromatography media fixed at its end with no dead volume. It is ideal for concentrating and purifying peptides or proteins in seconds prior to mass spectrometry, HPLC, capillary electrophoresis and other analytical techniques

5.1.2 MALDI-TOF MS

Peptide Mass Fingerprinting (PMF) analysis of the digested peptide samples was carried out on an Applied Biosystems Voyager DE-STR (Foster City CA) equipped with a nitrogen laser operated at 337 nm, 3 ns pulse. The instrument was operated in positive ion reflectron

mode. The samples were analyzed as follows:

To the MALDI plate 0.75 ul of Alpha-cyano-4-hydroxycinnamic acid (α-CHCA) matrix (5 mg/ml in 75% acetonitrile/ 0.1% TFA) and 0.75 ul of protein digest was added, mixed on the plate and air-dried under a gentle stream of warm air. The MALDI plates had previously been optimized for mass accuracy with the OptiPlate software in the Voyager 5.1 software (Applied Biosystems). PMF analysis of the digested proteins was done in the Automatic Control Mode with recalibrations every 4 samples using a mixture of Angiotensin 1 (M+H 1296.6853), ACTH 1-17 (M+H 2093.0867), and ACTH 18-39 (M+H 2465.1989), and when present, the spectra were internally calibrated with the autolytic fragment from trypsin (MH^+ 842.5100 m/z). 200 laser shots were averaged, and then smoothed, background corrected and converted to the monoisotopic values with Data Explorer (Applied Biosystems) The resulting peak lists were submitted to MASCOT for database searching.

5.2 In Vitro Oxidation of Ferritin and Transferrin Experiments

Horse Spleen Ferritin and Human serum Transferrin were purchased from Sigma and, in eight separate microcentrifuge tubes (Eppendorfs) they were treated, according to the method of Rudek et al. (2000) with two different concentrations of 50 uL hydrogen peroxide (10 and 20 uM) in 250 uL of 20 mM phosphate buffer pH 7.2. The tubes were incubated in a water bath at 37ºC as shown in Table 2.

Table 2: The different concentrations of H_2O_2 and the time of exposure of ferritin and transferrin to oxidative stress

Protein	**H_2O_2 Concentration**	**Time of Exposure**
Ferritin no. 1	10 µM	½ hour
no. 2	10 µM	3 hours
no. 3	20 µM	½ hour
no. 4	20 µM	3 hours
Transferrin no. 1	10 µM	½ hour
no. 2	10 µM	3 hours
no. 3	20 µM	½ hour
no. 4	20 µM	3 hours

500 µL of 10 mM EDTA (iron chelator) was added to each Eppendorf tube and the reaction tubes were transferred to ice. According the method of Conrad et al. (2000) with some modifications, the reactants were removed by dialysis for 16 hours at 4ºC against at least 125,000 volumes of 20 mM Tris/150 mM NaCl, pH 6.8. The first cycle of dialysis contained 10 mM EDTA.

The samples were then subjected to proteolytic digestion using trypsin, for subsequent protein identification or characterization.

5.2.1 Poroszyme method for Protein Digestion

The protocol used for (immobilized trypsin bead) digestion of ferritin and transferrin standards, as described in Section 5.1.1, was also applied to the *in vivo* experiments.

5.2.2 Protein Identification by Q-TOF LC-MS/MS

The digest was evaporated to dryness, then dissolved in 12 µl of 1% aqueous TFA, of which 6 µL were analyzed by LC-MS/MS using a capLC ternary HPLC system (Waters, Milford, MA, USA) interfaced to a Q-TOF Ultima Global hybrid tandem mass spectrometer fitted with a Z-spray nanoelectrospray ion source (Micromass, Manchester, UK). Solvent A and C were comprised of 0.2% formic acid in water, while solvent B consisted of 0.2% formic acid in acetonitrile. The peptide digest sample was loaded onto a C18 trapping column (Symmetry 300, 0.35 x 5 mm Opti-pak; Waters) and washed for 3 min using solvent C at a flow rate of 30 µL/min. The flow path was then switched using a 10-port rotary valve, and the sample eluted onto a C18 analytical column (PepMap, 75 µm x 15 cm, 3-µm particle size; LC Packings). Separations were performed using a linear gradient of 5:95% to 60:40 % B: A period of 43 min. The composition was then changed to 80:20 % B: A and held for 10 min to flush the column before re-equilibrating for 7 min at 0:100 % A: B. Mass calibration of the Q-TOF instrument was performed using a product ion spectrum of Glu-fibrinopeptide B acquired over the *m/z* range 50 to 1900. LC-MS/MS analysis was carried out using data dependent acquisition, during which peptide precursor ions were detected by scanning from *m/z* 400 to 1900 in TOFMS mode. Multiply charged (2+, 3+, or 4+) ions rising above predetermined threshold intensity levels were automatically selected for TOF-MS/MS analysis, by directing these ions into the collision cell where they were fragmented using low energy CID by

collision with argon. Product ion spectra were acquired over the *m/z* range 50-900. LC-MS/MS data were processed using *de novo* sequencing software package PEAKS to assign as many high abundance peaks in the spectrum as possible.

5.3 In Vitro Excess Iron Overload and Oxidation of Ferritin

These experiments were conducted according to the methods of Silva, D.et al. 1992; Conrad, C. et al. 2000, and Davies et al. 2003, with some modifications:

- 10 µL of Horse Spleen Ferritin (Sigma) were incubated in 700 uL of HEPES buffer pH 7.0 (8 preparations).
- 4 of these preparations were treated with 50 µL of 10 uM hydrogen peroxide, the other 4 were treated with 50 uL of 20 uM hydrogen peroxide.
- All preparations were incubated in a water bath at 37ºC for 3 hours.
- 50 µL of 0.5 mM ferrous ammonium sulfate was added to 4 preparations (2 with low hydrogen peroxide concentration and the other 2 had high hydrogen peroxide concentration).
- 200 µL of 0.5 mM ferrous ammonium sulfate was added to the other 4 preparations (2 with low hydrogen peroxide concentration and the other 2 with the higher hydrogen peroxide concentration).
- The 0.5 mM ferrous ammonium sulfate was added 10 times at 37ºC with 3 min interval for half hour.
- 4 out of those preparations underwent thermal denaturation by heating at 95ºC for 7 minutes followed by quick chilling on ice. However the other 4 did not undergo thermal denaturation.
- Reactants were removed by overnight dialysis at 4ºC against at least 125,000 volumes of 20 mM Tris/150 mM NaCl, pH 6.8.
- Samples were collected the next day and washed twice using 100 mM ammonium bicarbonate.
- The samples were analyzed by Q-TOF LC-MS/MS.

5.4 In Vitro Excess Iron Overload and Oxidation of Transferrin

5.4.1 Experimental Design

These experiments were conducted according to the methods of Chahine, JM. et al., 1996, 1999 and Davies, J. et al. 2003, with some modifications:

- 8 preparations were prepared by incubating 10 µl of serum transferrin in ~ 300 ul of 20 mM sodium bicarbonate for each.
- 4 out of these preparations were allowed to interact with only one equivalent of FNA_{c3}, the other 4 were allowed to interact with 2 equivalents of FNA_{c3}. The reaction mix was incubated for 4 hours 37ºC in a water bath.
- Half of the preparations were treated with 50 uL of 10 µM hydrogen peroxide, the other half was treated with 50 ul of 20 uM hydrogen peroxide.
- Half of the preparations were heated at 95ºC for 7 minutes, followed by quick chilling on ice for denaturation. The other half was not heated.
- Reactants were removed by dialysis for 16 hours at 4ºC against at least 125,000 volumes of 20 mM Tris/150 mM NaCl, pH 6.8.
- The samples were washed with ~ 250 µL of 100 uM ammonium bicarbonate after removal from the dialysis membranes.
- The proteins were digested into peptides and analyzed by Q-TOF LC-MS/MS using the same protocol mentioned above on page 41.

5.5 In Vivo Detection of Oxidative Modification in Plasma Samples of Patients of Hereditary Hemochromatosis (HH)

We used the methods of Michielsen, E. et al, 2005 Sisson, T. et al. 1990, a highly sensitive immunoprecipitation technique for extracting and concentrating proteins such as ferritin and transferrin in the plasma samples from patients with HH with a few modifications.

5.5.1 Immunoprecipitation Protocol

- The reactions were performed at room temperature.
- 100 mg of protein-G agarose (Sigma) was washed once with phosphate-buffered saline (PBS), pH 7.0 and then blocked with 250 µL of 1 g/L bovine serum albumin (BSA) (Sigma) in PBS for 60 min.
- A mixture of anti-ferritin and anti-transferrin antibodies (Sigma) [2 µL of each of a concentration of 55 µg of each antibody]. This mixture was mixed with PBS containing 1g/L BSA.
- Upon thorough mixing, the mixture was mixed with agarose beads and rotated for 1 h.
- After binding the antibodies, the beads were washed twice with excessive PBS.
- Antibodies were cross-linked to protein G-agarose by the addition of 200 mM triethanolamine (Pharmacia Biotech) in PBS to which 50 mM dimethyl pimelimidate (Sigma) was added directly before use (final pH 8.6) with rotation for 30 min.
- Cross-linking and washing were repeated twice to improve cross-linking efficiency.
- To each eppendorf, 250 µL of plasma, 100 µL of 6 M urea and 150 µL PBST (PBS containing 0.2 mL/L Tween 20) and protein G-agarose beads were added, then rotated for 90 min and washed twice with PBST.
- Elution was performed by heating at 56ºC for 20 min in 100 µL of 1 M glycine, pH 3.0.

- Samples were centrifuged and supernatants were kept for analysis.
- Samples for electrophoresis were prepared by adding 20 µL of sample buffer (40 mM tris/HCl, 33 g/L SDS, 500 ml/L glycerol and Bromophenol Blue) to 50 µL of the eluate.

5.5.2 SDS-Polyacrelamide Gel Electrophoresis (Linear Slab Gel)

A rapid method was used for qualifying, comparing and characterizing proteins which separates proteins based primarily on their molecular weights as described by Laemmli, 1970.

- According to manufacturer's (e.g. Rad Mini-Gel). The bottom of both gel plate and spacer were perfectly flushed against a flat surface before tightening the clamp assembly to avoid any leak.
- 10% separating gel was prepared by adding 4.1 mL distilled water, 2.5 mL of 1.5 M Tris-HCl (pH 8.8), 3.3 mL of acrylamide/bis-acrylamide, 50 µL of 20% SDS and 50 µL of 10% ammonium persulfate. 15 µL of TEMED was added right before pouring the separating gel solution onto the gel sandwich using a pipette.
- About 15 min were allowed for polymerization.
- 4% stacking gel was prepared; 3.075 mL distilled water, 1.25 mL of 0.5 M Tris-HCl pH 6.8, 25 µL of 20% SDS, 0.67 mL of 30% acrylamide/bis-acrylamide, and 25 µL of 10% ammonium persulfate. 10 µL of TEMED was added right before pouring the stacking gel solution onto the gel sandwich.
- The stacking gel was added onto the gel sandwich until the solution reached the top of the front plate.
- A comb was carefully inserted into the gel sandwich until the bottom of the teeth reached the top of the front plate.

- About 30 min were allowed for the stacking gel to polymerize , followed by careful removal of the comb.
- Using a Hamilton syringe, 30 µL of the sample solution was introduced into the wells, avoiding air bubbles and leaving reasonable spaces among wells.
- Within the Mini-Protean electrophoresis chamber , the gel was placed and a running buffer was added to the inner and outer reservoirs.

NOTE: To prepare 1 liter of running buffer pH 8.3, 15 g of Tris Base was added to 72 g Glycine and 5 g SDS in distilled water to a final volume of 1 liter.

- A molecular mass standard was run in parallel.
- The gel was run at 120 V for approximately 40-50 min using a Power-Pac 300 power supply (Biorad).
- After turning the power off, the electrodes as well as the spacer and the gel plate were removed.
- The gel was completely soaked in Coomassie Blue Stain for 10-20 min.
- About 50 mL of Coomassie destain was added overnight after pouring the stain out.
- The appropriate bands were excised the following day for digestion by proteolytic enzymes (Trypsin).

5.5.3 Trypsin Digestion

- **Coommasie de-stain:**

Samples were dispensed with 50 µL of 100 µM ammonium bicarbonate and 50 µL acetonitrile, followed by a10 min incubation at room temperature. 150 µL from each well was aspirated. Another 50 µL of ammonium bicarbonate and acetonitrile were

added followed by another 10 min incubation. 95 µL from each well were aspirated.

- **Dehydration:**

  Samples were treated with 50 µL acetonitrile, and allowed to stand for 5 min. 45 µL were aspirated and followed by 15 min incubation.

- **Reduction:**

  50 µL 10 mM Dithiothreitol (DTT) were added and allowed to stand for 30 min.

- **Alkylation:**

  50 µL 55 mM iodoacetamide was added and allowed to stand for 20 min. Another 100 µL acetonitrile was added and allowed to stand for 5 min waiting. 195 µL were aspirated from each well.

- **Wash:**

  50 µL ammonium bicarbonate was added twice, followed by 10 min and 5 min incubation. 120 µL were aspirated.

- **Dehydration I:**

  50 µL acetontrile was dispensed followed by 5 min waiting. 45 µL were aspirated from each well.

- **Dehydration II:**

  50 µL acetonitrile was added, followed by 5 min incubation. 75 µL from each well were aspirated followed by another 5 min incubation. Heater tiles were switched off and allowed to stand for 30 min.

- **Preparation of Trypsin:**

  Water as well as 100 mM ammonium bicarbonate were dispensed to each trypsin vial with mixed aspiration and dispensation. Final concentration of trypsin was 6 ng/µL.

- **Digestion:**

  25 µL trypsin was added, followed by 30 min incubation. Heater tiles were switched on and allowed to stand for about 4.5 hours. Heater tiles were switched off.

- **Extraction I:**

  30 µL of 1% formic acid and 2% acetonitrile solution were dispensed, followed by 30 min incubation.

- **Peptide Transfer:**

  15 µL were aspirated from each well and dispensed to cooled PCR plate.

- **Extraction II:**

  12 µL formic acid and 12 µL acetonitrile solution were dispensed, followed by 30 min incubation. 15 µL were aspirated from each well and dispensed to cooled microtiter plate.

- **Extraction III:**

  Same as extraction II.

  Note: Fixed tips were used for all reagent conditions:

  - 200 µL MBP tips were used for all aspirations to waste. Used 200 µL MBP tips were discarded.
  - 20 µL MBP tips were used for peptide transfer. Used ones were discarded.

5.5.4 Analysis using MALDI-TOF and Q-TOF LC-MS/MS

The same protocol mentioned above on pages 37 and 40 was used.

5.6 Specrophotometric Assay for the Detection of Protein Carbonyls in the Plasma Samples of Patients With Hereditary Hemochromatosis

The methods used were those of Levine, R. et al. 1990, 1994; Reznick, A. et al. 1994 and Shacter, E. 2000. The most convenient procedure to detect and quantitate the carbonyl content in protein preparation is the reaction with 2,4-dinitrophenylhydrazine (DNPH). DNPH reacts with protein carbonyls, forming a Schiff base to produce the corresponding hydrazone, which can be analyzed spectrophotometrically at a wavelength of 370 nm.

5.6.1 Assay Range

This assay works best when the samples have protein concentration in the range of 1.0-10 mg/mL.

5.6.2 Pre-Assay Preparation and Stock Solutions

1. Hydrochloric acid: 2.5 M HCl was prepared to resuspend the DNPH.
2. 2,4-dinitrophenylhydrazine (DNPH): 10 mM was resuspended in 2.5 M HCl. DNPH is stable for one week at 4°C in the dark.
3. Trichloroacetic Acid Solutions (TCA): 10% and 20% solutions were prepared for the precipitation of the plasma proteins. Both solutions are stable at room temperature for at least one week.
4. Guanidine Hydrochloride: 6 M of Guanidine HCl were prepared for redissolving of the protein.
5. Ethanol/Ethyl Acetate: 1:1 ethanol / ethyl acetate were prepared to help remove the free reagents from the protein preparations.

5.6.3 Plasma Samples Preparation

A 5.0 mL blood sample was collected from each untreated HH patient as well as one normal individual as a control, in heparinized tubes. Six patients who gave consent were

recruited from HH patients attending the Department of Internal Medicine (Gastroenterology and Hepatology Division) at the Royal University Hospital in Saskatoon, Saskatchewan, Canada. The blood samples were centrifuged upon withdraw at 2000-x g for about 15 min to collect plasma which was stored on ice until use or frozen at -20ºC.

Table 3: Comparison of Control and HH patients. ND*: Not determined, Sex*: Males are predominant.

	Control	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age	44	49	41	41	40	63	56
Sex*	M	F	M	M	M	M	F
Serum ferritin, ng/ml (N: 20 –250)	55	182	1517	195	767	870	310
Serum transferrin saturation, % (N: 17 – 50)	22	54	93	ND*	82	76	78
Serum iron, µM/L (N: 11.6 – 31.3)	ND	25	52	ND	36	39	ND
AST, U/L (N: 8 – 20)	10	24	114	49	22	38	ND
ALT, U/L (N: 8 – 20)	12	30	158	59	36	51	ND
Clinical information	ND	Two copies of C282Y & Fatigue	One copy of H63D & HCV positive	C282Y/ H63D compound heterozygote	Two copies of C282Y & fatty liver	C282Y/ H63D compound & fatty liver.	ND

5.6.4 Procedure

- 100 µL of each sample (including the control) was transferred to 2.0 mL plastic tubes.
- 400 µL of DNPH was added to sample tubes, while 400 µL of 2.5 M HCl were added to the control.
- All tubes were incubated in the dark at room temperature for one hour with vortexing each tube every 15 min during the incubation.

- 0.5 mL of 20% TCA was added to each tube. Tubes were incubated on ice for 5 min.
- Tubes were centrifuged at 10,000-x g for 10 min.
- The supernatants were discarded and the pellets were resuspended in 0.5 mL of 10% TCA, and reincubated incubated on ice for 5 min.
- Tubes were centrifuged at 10,000-x g for 10 min.
- The supernatants were discarded and the pellets were resuspended in 0.5 mL of (1:1) ethanol/ethyl acetate with thorough vortexing and centrifugation at 10,000 x g for 10 min.
- Last step was repeated twice.
- After the final wash, protein pellets were resuspended in 0.5 mL of guanidine HCl and heated for 15 min at 37°C.
- Tubes were centrifuged at 10,000 x g for 10 min to remove the insoluble materials.
- The UV/Visible Spectrophotometer (Pharmacia Biotech) was used to obtain the spectrum by reading samples against 2.5 M HCl as a blank.
- The carbonyl content was measured from the maximum absorbance (370 nm) using a molar absorption coefficient of 22,000 M^{-1} cm^{-1}.

5.6.5 Determination of Carbonyl Content

- The average absorbance of each sample and control were calculated.
- The average absorbance of the control was subtracted from the average absorbance of the samples to give the **Corrected Absorbance (CA)**.
- The concentrations of the carbonyls were determined by inserting the **CA** into the following equation:

Protein Carbonyl (nmol/ml) = [(CA)/(22,000 $M^{-1}cm^{-1}$)](500 µL/200µL)

6.0 Results

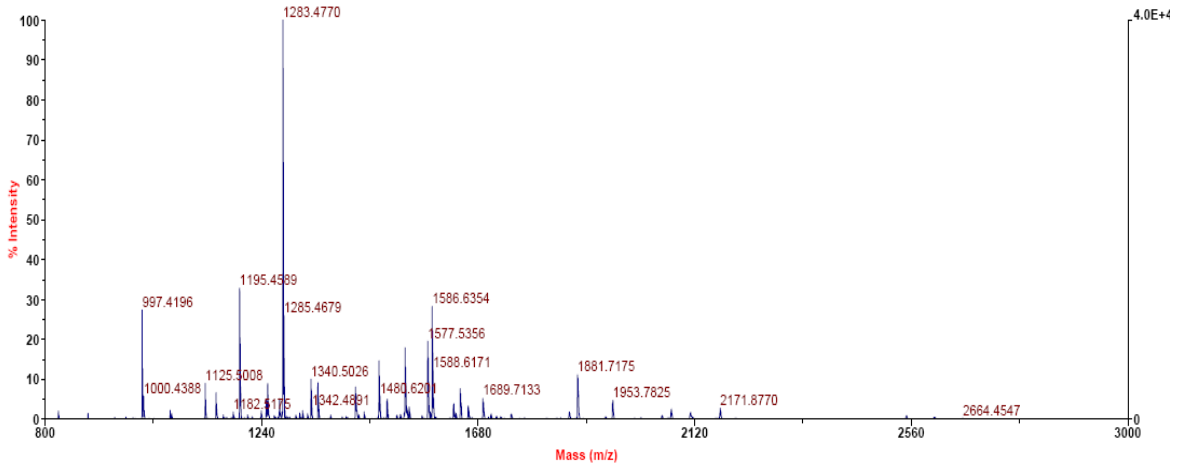
6.1 Results from 'gel-free' protein digest experiments

Transferrin digest provided enough tryptic peptides, and in sufficient quantities, to easily identify the protein by peptide mass fingerprinting using MALDI-TOF MS as shown in Fig.5 (A). Analysis of modified (and unmodified) peptides from endogenous levels of transferrin should therefore be straightforward using the Q-TOF instrument for LC-MS/MS analysis (which is required to identify and locate modifications due to oxidation).

Ferritin was not identified by peptide mass fingerprinting from amount of material used, although the MALDI-TOF spectrum shown in Fig.5 (B) did contain one or two weak ions that might have corresponded to tryptic peptides.

(A) Transferrin

MALDI-TOF MS spectrum of immobilized trypsin digest



MASCOT search result (SwissProt database)

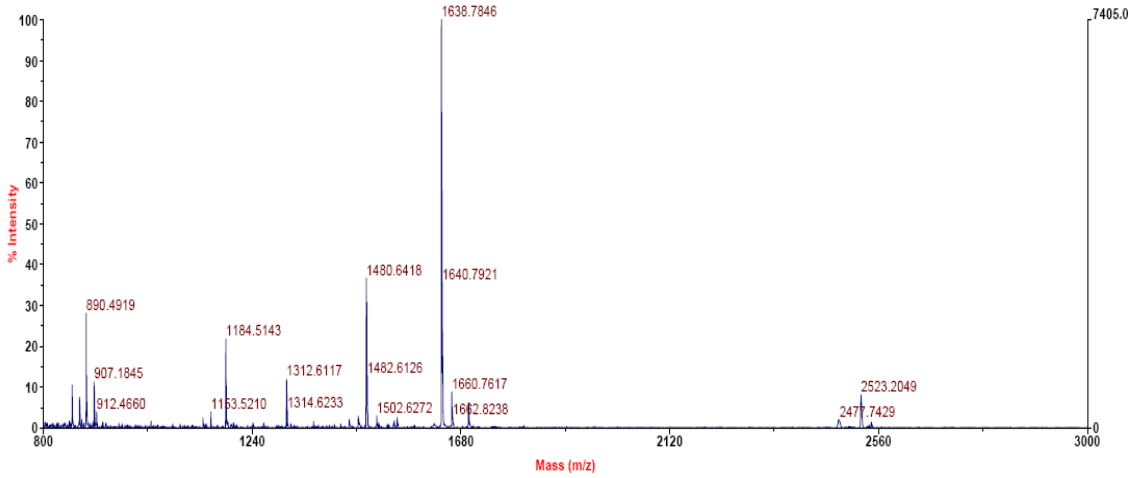
TRFE_HUMAN Mass: 79280 Score: 161 Expect: 1.4e-12 Queries matched: 20
Serotransferrin precursor - Homo sapiens (Human)

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Peptide
1000.4388	999.4315	999.4913	-59.79	669	676	0	K.YLGEEYVK.A
1125.5008	1124.4935	1124.5648	-63.38	61	69	1	K.KASYLDCIR.A
1195.4589	1194.4516	1194.5352	-70.01	363	371	0	K.WCALSHHER.L
1249.5274	1248.5201	1248.5986	-62.87	454	464	0	K.SASDLTWDNLK.G
1276.5313	1275.5240	1275.6248	-78.99	300	310	0	K.EFQLFSSPHGK.D
1283.4770	1282.4697	1282.5618	-71.82	531	541	0	K.EGYYGYTGAFR.C
1354.5351	1353.5278	1353.6235	-70.65	577	587	0	K.DYELLCLDGTR.K
1434.6062	1433.5989	1433.7150	-80.99	454	466	1	K.SASDLTWDNLKGK.K
1478.6157	1477.6084	1477.7275	-80.59	332	343	0	K.MYLGYEYVTAIR.N
1494.6200	1493.6127	1493.7224	-73.44	332	343	0	K.MYLGYEYVTAIR.N + Oxidation (M)
1531.5557	1530.5484	1530.6807	-86.39	684	696	0	K.CSTSSLLEACTFR.R
1539.5886	1538.5813	1538.7035	-79.40	240	251	0	R.DQYELLCLDNTR.K
1577.5356	1576.5283	1576.6504	-77.44	495	508	0	R.FDEFFSEGCAPGSK.K
1586.6354	1585.6281	1585.7671	-87.62	588	600	0	R.KPVEEYANCHLAR.A
1629.7001	1628.6928	1628.8086	-71.09	108	121	0	K.EDPQTFYYAVAVVK.K
1659.6239	1658.6166	1658.7756	-95.85	683	696	1	R.KCSTSSLLEACTFR.R
1689.7133	1688.7060	1688.8417	-80.33	259	273	0	K.DCHLAQVPSHTVVAR.S
1881.7175	1880.7102	1880.8687	-84.23	237	251	1	K.ADRDQYELLCLDNTR.K
1952.7659	1951.7586	1951.9309	-88.26	572	587	1	K.NLNEKDYELLCLDGTR.K
2071.7751	2070.7678	2070.9204	-73.70	434	452	0	K.SDNCEDTPEAGYFAVAVVK.K

No match to: 750.3351, 779.3498, 802.4512, 821.4295, 995.5164, 1014.5571, 1128.6240, 1147.4740, 1232.5846, 1252.4817, 1281.4658, 1296.6485, 1340.5026, 1430.5912, 1493.6696, 1496.6875, 1529.6945, 1605.8279, 1634.5853, 1643.6590, 1669.7307, 1766.7946, 1791.7772, 2113.9599, 2164.0891, 2170.8459

(B) Ferritin

MALDI-TOF MS spectrum of immobilized trypsin digest



MASCOT search result (SwissProt database)

FRIL_HORSE Mass: 20079 Score: 90 Expect: 0.00028 Queries matched: 6

Ferritin light chain - Equus caballus (Horse)

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Peptide
890.4916	889.4843	889.5385	-60.92	20	26	0	R.LVNLYLR.A
1184.5138	1183.5065	1183.5768	-59.36	145	154	0	K.MGDHLTNIQR.L
1312.6111	1311.6038	1311.6717	-51.78	144	154	1	K.KMGDHLTNIQR.L
1480.6421	1479.6348	1479.6954	-40.91	7	19	0	R.QNYSTEVEAAVNR.L
1638.7847	1637.7774	1637.8413	-39.01	155	169	0	R.LVGSQAGLGEYLFER.L
2522.1223	2521.1150	2521.2159	-40.00	77	98	0	R.ALFQDLQKPSQDEWGTTLDAMK.A

No match to: 860.9844, 876.4510, 907.1852, 1623.2353, 1660.7599, 1695.7652, 2475.2439, 2477.6827

Fig.5: MALDI-TOF MS spectra of immobilized trypsin (Poroszyme) digests and MASCOT peptide mass fingerprint (PMF) using SwissProt databases for (A) Transferrin (6 μg) and (B) Ferritin (60 ng).

6.2 Oxidized and unmodified peptides matching human transferrin and ferritin

Data were acquired by Q-TOF LC-MS/MS analysis of immobilized trypsin digests of proteins oxidized *in vitro*.

Database searches were performed using PEAKS software and the following modifications:

- oxidation of M, H, or W by a single oxygen atom (OxM, OxH, OxW)
- oxidation of M, H, or W by two oxygen atoms (OOM, OOH, OOW)
- oxidation of W to Kynurenine (KyW)
- oxidation of W to 3-hydroxy Kynurenine (OHKyW)

Oxidized peptides that give a significant match with human transferrin or ferritin are highlighted in red, may be used for targeted LC-MS/MS analysis in future experiments involving animal and human tissue and plasma samples.

6.2.1 Transferrin

6.2.1.1 Oxidized peptides that provided significant matches (≥50 %) with human transferrin (i.e. targets for LC-MS/MS analysis)

Table 4 shows oxidized and unmodified peptides matching human transferrin showing different m/z,charge, mr (calc) and score for different peptides.

Transferrin Sample 003

gi|6980467 more... Chain A, Human Transferrin N-Lobe Mutant H249e

Mass: 36789.996 **Score:** 95.89% Coverage: 44.91%

Mz	Charge	Mr (calc)	Start	End	Score	Peptide
489.731	2	959.4712	197	206	60.78%	DGAGDVAFVK
489.778	2	959.4712	197	206	8.96%	DGAGDVAFVK
489.7922	2	959.4712	197	206	37.95%	DGAGDVAFVK
544.8204	4	2157.016	236	254	20.25%	DEYKDC(OxH)LAQVPSETVVAR
544.8688	4	2157.016	236	254	1.18%	DEYKDC(OxH)LAQVPSETVVAR
568.651	4	2252.1226	258	276	1.09%	GKEDLIWELLNQAQE(OxH)FGK
580.0648	4	2298.128	260	278	6.76%	EDLI(KyW)ELLNQAQEHFGKDK
625.6363	3	1855.8853	264	278	2.48%	(OOW)ELLNQAQEHFGKDK
680.1284	4	2699.3125	254	276	3.86%	RS(OxM)GGKEDLI(OxW)ELLNQAQEHFGK
698.1269	4	2770.3384	255	278	82.78%	S(OxM)GGKEDLIWELLNQAQEHFGKDK
740.8903	4	2942.4343	254	278	2.64%	RS(OxM)GGKEDLI(OxW)ELLNQAQEHFGKDK
747.8765	2	1475.7119	313	324	99%	(OxM)YLGYEYVTAIR
755.1469	4	2997.4766	253	278	1.33%	ARSMGGKEDLIWELLNQAQE(OxH)FGKDK
762.4193	3	2267.152	124	143	1.3%	RSAG(OxW)NIPIGLLYCDLPEPR
762.4345	3	2267.152	124	143	72.46%	RSAG(OxW)NIPIGLLYCDLPEPR
781.4538	3	2324.1736	123	143	20.63%	GRSAG(OxW)NIPIGLLYCDLPEPR
799.1362	4	3174.5515	206	232	50.38%	KHSTIFENLANKADRDNYELLCLDNTR
827.6469	4	3289.615	205	232	46.02%	VK(OxH)STIFENLANKADRDNYELLCLDNTR
989.3256	4	3934.9993	51	88	99%	AIAANEADAVTLDAGLVYDAYLAPNNLKPVVAEFYGSK

1065.1929	3	3174.5515	206	232	1.17%	KHSTIFENLANKADRDNYELLCLDNTR
1318.7422	3	3934.9993	51	88	17.6%	AIAANEADAVTLDAGLVYDAYLAPNNLKPVVAEFYGSK

Table 4 shows a significant high score of oxidized Met and Trp, 82.8% 99% and 72% respectively as highlighted in red. The m/z column indicates the mass to charge ratio, while the mr(calc) column indicates the corresponding total mass of the amino acid residues in the detected peptide. The start column indicates the first amino acid residue of the peptide matching while the end column indicates the last amino acid residue of the matching peptide.

6.2.2 Ferritin

6.2.2.1 Oxidized peptides that provided significant matches (≥ 50 %) with horse spleen ferritin (i.e.targets for LC-MS/MS analysis)

Table 5 shows oxidized and unmodified peptides matching horse spleen ferritin showing different mz, charge, mr (calc) and score for different peptides.

Mass: 19814.104 **Score:** 100% Coverage: 50.57%

Mz	Charge	Mr(calc)	Start	End	Score	Peptide
437.7761	2	855.445	53	59	60.33%	ELAEEKR
445.7761	2	871.52795	19	25	99%	LVNLYLR
445.8241	2	871.52795	19	25	92.13%	LVNLYLR
481.8107	2	943.5127	76	83	35.45%	ALFQDLQK
494.2751	3	1461.6848	6	18	97.22%	QNYSTEVEAAVNR
538.3058	2	1056.52	9	18	99%	STEVEAAVNR
547.0021	3	1619.8307	154	168	99%	LVGSQAGLGEYLFER
584.0082	3	1730.87	4	18	73.18%	IRQNYSTEVEAAVNR
619.8489	2	1219.5833	8	18	99%	YSTEVEAAVNR
676.8781	2	1333.6262	7	18	56.02%	NYSTEVEAAVNR
713.9045	2	1407.6782	156	168	95.04%	GSQAGLGEYLFER
740.8243	2	1461.6848	6	18	99%	QNYSTEVEAAVNR
740.9094	2	1461.6848	6	18	99%	QNYSTEVEAAVNR
763.4441	2	1506.7466	155	168	86.25%	VGSQAGLGEYLFER
784.8048	3	2333.2126	6	25	95.91%	QNYSTEVEAAVNRLVNLYLR
819.9608	2	1619.8307	154	168	99%	LVGSQAGLGEYLFER
819.9691	2	1619.8307	154	168	93.72%	LVGSQAGLGEYLFER
819.9781	2	1619.8307	154	168	94.67%	LVGSQAGLGEYLFER
819.9949	2	1619.8307	154	168	99%	LVGSQAGLGEYLFER
819.995	2	1619.8307	154	168	98.9%	LVGSQAGLGEYLFER
819.9955	2	1619.8307	154	168	98.26%	LVGSQAGLGEYLFER

819.9958	2	1619.8307	154	168	99%	LVGSQAGLGEYLFER
819.9989	2	1619.8307	154	168	95.8%	LVGSQAGLGEYLFER
820.0275	2	1619.8307	154	168	84.08%	LVGSQAGLGEYLFER
846.7948	3	2519.2002	76	97	81.05%	ALFQDLQKPSQDEWGTTLDA(OxM)K
855.2383	4	3399.7495	75	104	22.31%	RALFQDLQKPSQDEWGTTLDA(OxM)KAAIVLEK
860.4593	3	2560.2842	82	104	4.13%	QKPSQDE(OHKyW)GTTLDAMKAAIVLEK
865.797	3	2576.279	82	104	5.29%	QKPSQDE(OHKyW)GTTLDA(OxM)KAAIVLEK
912.8155	3	2717.3582	81	104	1.51%	LQKPSQDE(OOW)GTTLDA(OOM)KAAIVLEK
936.4847	3	2788.3953	80	104	2.2%	DLQKPSQDE(OHKyW)GTTLDAMKAAIVLEK

Table 5 shows a significant score (81.05%) for oxidized Met at a single O atom, as highlighted in red. This table also indicates that the doubly, triply and the quadruply charged ions shown in the m/z column are 18 Daltons larger than those shown in the Mr (calc) when the m/z is multiplied by the number of the charges. This is because PEAKS does not account for the absence of H_2O (H^+ at C- terminal and OH^- at N-terminal) when using the masses of the component amino acid residues to determine Mr(calc).

6.2.3 Oxidized peptides that provided significant matches with human transferrin (i.e. targets for LC-MS/MS analysis)

Table 6 Summary of the significant matches with human transferrin including only the oxidized peptides from all samples.

606.3192	2	1192.5295	123	132	73.24%	DSGFQ(OxM)NQLR
666.6204	4	2645.3496	251	273	71.4%	RKPVDEYKDC(OxH)LAQVPSHTVVAR
698.1269	4	2770.3384	255	278	82.78%	S(OxM)GGKEDLIWELLNQAQEHFGKDK
747.8765	2	1475.7119	313	324	99%	(OxM)YLGYEYVTAIR
797.4498	2	1574.7915	476	489	92.85%	TAGWNIP(OxM)GLLYNK
762.4345	3	2267.152	124	143	72.46%	RSAG(OxW)NIPIGLLYCDLPEPR
846.7921	3	2519.2002	76	97	85.79%	ALFQDLQKPSQDEWGTTLDA(OxM)K

Note: most of the high scoring peptides are oxidized by attachment of a single O at Methionine.

Figure 6 shows the annotated MS/MS of *m/z* 606.3192 *in vitro* sample MOH070505_002. Strong y-type ions are shown (y, y2, y4, y6 and y7) and x9. Both x and y-type ions retain the C-terminal residues. x-type ion is only formed predominantly in high energy dissociation experiments at C-terminal. Only one a-type ion (a2-type) is shown that retains the N-terminal residue. The abundance of y-type ions is typical of low energy CID experiments.

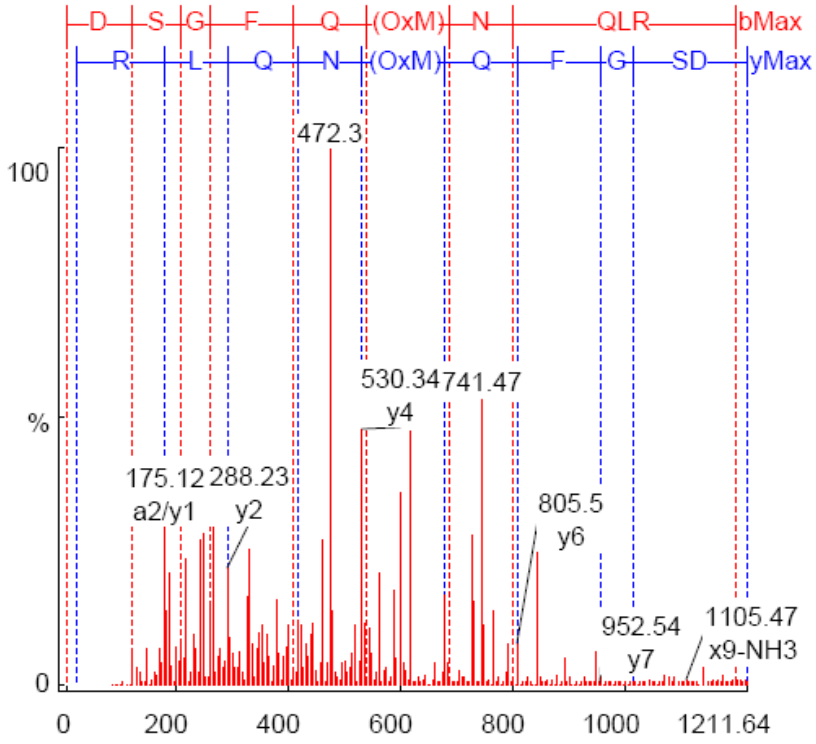


Fig.6: MS/MS spectrum of *m/z* 606.3192 from *in vitro* sample MOH070505_002, annotated by PEAKS after database matching against transferrin (Accession gi|31415705; 73.2% confidence).

6.2.4 Oxidized peptides that provided significant matches with horse spleen ferritin (i.e. targets for LC-MS/MS analysis)

Table 7 Summary of oxidized peptides that provided significant matches with horse spleen ferritin including only the oxidized peptides from all samples.

846.7972	3	2519.2002	76	97	89.77%	ALFQDLQKPSQDEWGTTLDA(OxM)K

6.3 Database search results for ferritin and transferrin LC-MS/MS data processed using PEAKS

As described in Materials and Methods Section; oxidative stress and excess iron load were induced in both horse spleen ferritin and human serum transferrin.

KEY: (OxM) = methionine oxidized with one O

(OOM) = methionine oxidized with two O

(OxH) = histidine oxidized with one O

(OOH) = histidine oxidized with two O

(OxW) = tryptophan oxidized with one O

(OOW) = tryptophan oxidized with two O

N.B. – this list shows *only* the **oxidized tryptic peptides** that matched ferritin or transferrin. The same peptide sequence may appear more than once in each sample, either because it was detected as two peptide ions of different charge, or because two or more versions of that peptide containing different modifications were matched.

Oxidized peptides that match with reasonable confidence (i.e. Score = 50% or better) are highlighted in red.

Oxidized peptides that have a relatively low score (< 50%) are marked in blue, and should be disregarded *unless* they are detected in more than one sample.

6.3.1 Ferritin

6.3.1.1 Native Ferritin with 20 μM H_2O_2

Table 8 shows different mz, charges, mr (calc) and scores for different peptides of native ferritin oxidized with 20 uM H_2O_2. OxM shows a significant confidence score of 52.47-79.4% at a single O as highlighted in red. However, relatively low scoring (OxM and OOH), highlighted in blue, are also shown.

Mass: 19844.473 **Score:** 88.03% Coverage: 33.33%

Mz	Charge	Mr (calc)	Start	End	Score	Peptide
400.9225	3	1199.5718	144	153	79.41%	(OxM) GDHLTNIQR
600.8906	2	1199.5718	144	153	52.47%	(OxM) GDHLTNIQR
616.8919	2	1231.5615	144	153	35.72%	(OxM) GD(OOH)LTNIQR
770.4661	2	1538.6824	40	52	7.31%	DDVALEGVC(OOH)FFR

6.3.1.2 Denatured Ferritin with 20 μM H_2O_2

Table 9 shows different mz, charges, mr (calc) and scores for different peptides of denatured ferritin oxidized with 20 uM H_2O_2. OxH and OxM show a significant confidence score of 76.8% and 99% at a single O, as highlighted in red, respectively. A relatively non significant, low score (5.19%) of oxidized Met and His at 2 Os are also shown, as highlighted in blue.

Mz	Charge	Mr (calc)	Start	End	Score	Peptide
400.8713	3	1199.5718	144	153	76.83%	MGD(OxH)LTNIQR
846.7748	3	2537.2107	76	97	99%	ALFQDLQKPSQDEWGTTLDA(OxM)K
1117.5837	3	3349.745	140	168	5.19%	LIKK(OOM)GD(OOH)LTNIQRLVGSQAGLGEYLFER

6.3.2 Transferrin

6.3.2.1 Native Transferrin with 20 μM H_2O_2

Table10 shows different mz, charges, and score of different peptides of native transferrin oxidized with 20 uM H2O2. Confidence scores of oxidized Met, and Trp at a single O were detected with an average of 84.5 – 99 and 93.4 – 98.3%, as highlighted in red, respectively. Oxidized His is detected in this sample with a significant score of 81.6–91.4% only at (OOH) i.e. no significant score for oxidized His at a single O (OxH) was detected. As well, no significant score for oxidized Met at 2 O s (OOM) was detected in this sample. Oxidized Trp at 2 O s (OOW) is shown in one peptide with a score of 99%, as highlighted in red. As highlighted in blue, some low confidence scores (< 50%) were also detected at some other locations.

Mass: 77079.92 **Score:** 99.88% Coverage: 36.39%

Mz	Charge	Mr (calc)	Start	End	Score	Peptide
453.7552	2	905.42426	316	323	87.58%	DSA(OOH)GFLK
498.7909	2	995.51483	601	609	91.42%	APN(OOH)AVVTR
526.9611	3	1577.8049	453	466	20.4%	KSASDLT(OxW)DNLKGK
584.2852	2	1166.4961	38	46	14.11%	CQSFRDH(OxM)K
633.3508	2	1264.5935	454	464	93.41%	SASDLT(OxW)DNLK
641.3419	2	1280.5884	454	464	99%	SASDLT(OOW)DNLK
642.3898	3	1923.9729	300	315	81.6%	EFQLFSSP(OOH)GKDLLFK
747.9221	2	1493.7224	332	343	99%	(OxM)YLGYEYVTAIR
797.3975	2	1592.802	476	489	84.5%	TAGWNIP(OxM)GLLYNK
805.4689	2	1608.7969	476	489	93.01%	TAG(OxW)NIP(OxM)GLLYNK
849.4998	3	2545.227	274	295	97.59%	S(OxM)GGKEDLIWELLNQAQEHFGK
1043.6183	2	2085.0166	279	295	98.33%	EDLI(OxW)ELLNQAQEHFGK
1051.6409	2	2101.0115	279	295	15.66%	EDLIWELLNQAQE(OOH)FGK

Figure 7 shows the annotated MS/MS of *m/z* 747.9221*in vitro* samples DF-3. Strong y-type ions are shown (y4, y6, y7, y9 and y11). y-type ions retain the C-terminal residues. Only one a-type ion (a2-type), one b-type ion (b2) and one c-type ion (c2-type) are shown that retains the N- terminal residue. The peptide (OxM)YLGYEYVTAIR (m/z 747.9221) was found in virtually all the *in vitro* samples of oxidized transferrin, and was the peptide that best matched transferrin in the database searches (with 99%) confidence. This would be the best example of a peptide that demonstrates oxidation of transferrin *in vitro*.

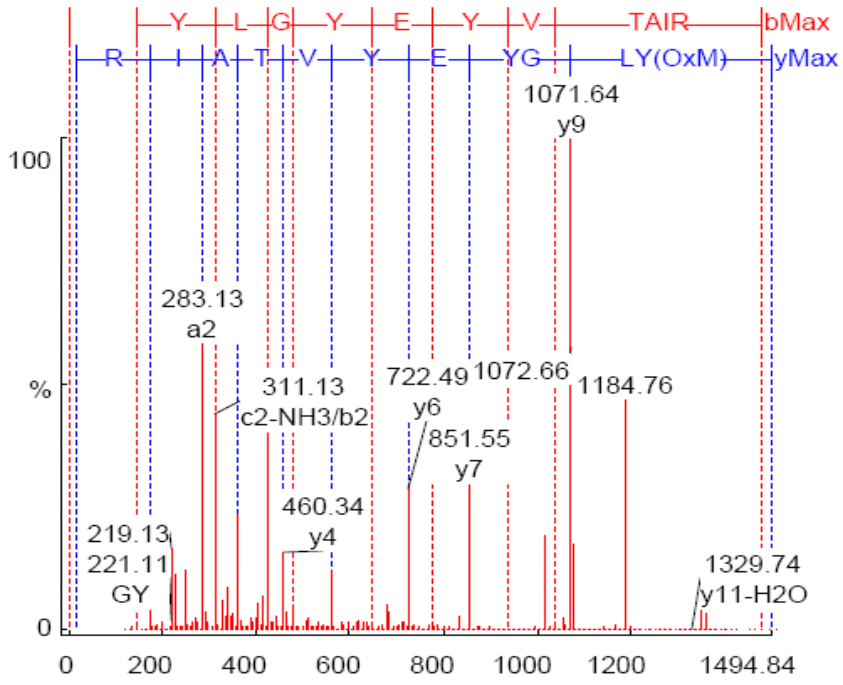


Fig.7: MS/MS spectrum of *m/z* 747.9221 from *in vitro* sample DF-3, annotated by PEAKS after database matching against transferrin (Accession gi|62897069; 99% confidence).

Figure 8 shows the annotated MS/MS of *m/z* 797.5536 *in vitro* samples NT-4. y-type ions are shown (y_2 , y_3). y-type ions retain the C-terminal residues. No b-type ions were annotated.

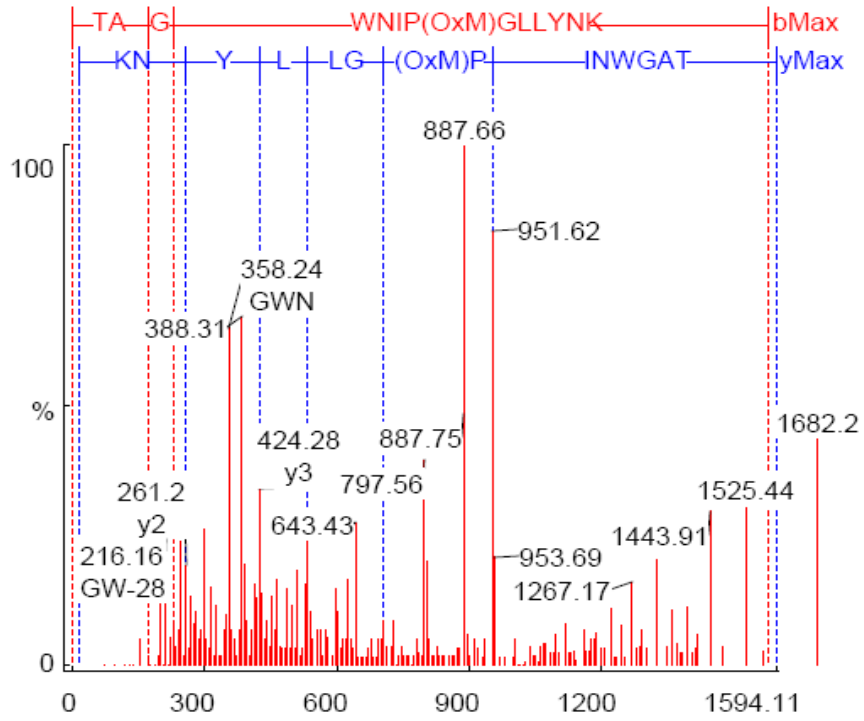


Fig.8: MS/MS spectrum of *m/z* 797.5536 from *in vitro* sample NT-4, annotated by PEAKS after database matching against transferrin (Accession gi|15021381; 65.3% confidence).

6.3.2.2 Denatured transferrin with 20 μM H_2O_2

Table 11 shows high significantly scores of oxidized Trp at both single and 2 O (s) with a score of 99%, as highlighted in red. A significant score of 83.6 – 98% is also shown for oxidized Met at a single O, as highlighted in red. A relatively non significant score of 5% was detected with oxidized His at both single and 2 O (s), as highlighted in blue.

Mass: 77049.945 **Score:** 99.08% Coverage: 28.65%

Mz	Charge	Mr(calc)	Start	End	Score	Peptide
580.3556	2	1158.5928	133	143	5.32%	GKKSC(OxH)TGLGR
633.4001	2	1264.5935	454	464	99%	SASDLT(OxW)DNLK
641.3895	2	1280.5884	454	464	99%	SASDLT(OOW)DNLK
642.4209	3	1923.9729	300	315	53.05%	EFQLFSSP(OOH)GKDLLFK
747.9763	2	1493.7224	332	343	98.02%	(OxM)YLGYEYVTAIR
797.4877	2	1592.802	476	489	83.57%	TAGWNIP(OxM)GLLYNK
993.5349	4	3969.9165	226	258	4.89%	(OOH)STIFENLANKADRDQYELLCLDNTRKPVDEYK

Figure 9 shows the annotated MS/MS spectra of *m/z* 633.4001 from *in vitro* sample DT-4. Strong y-type ions are shown (y2, y3, y4 and y8). Only one b-type ion (b4-type) and one c-type ion (c4-type) are shown.

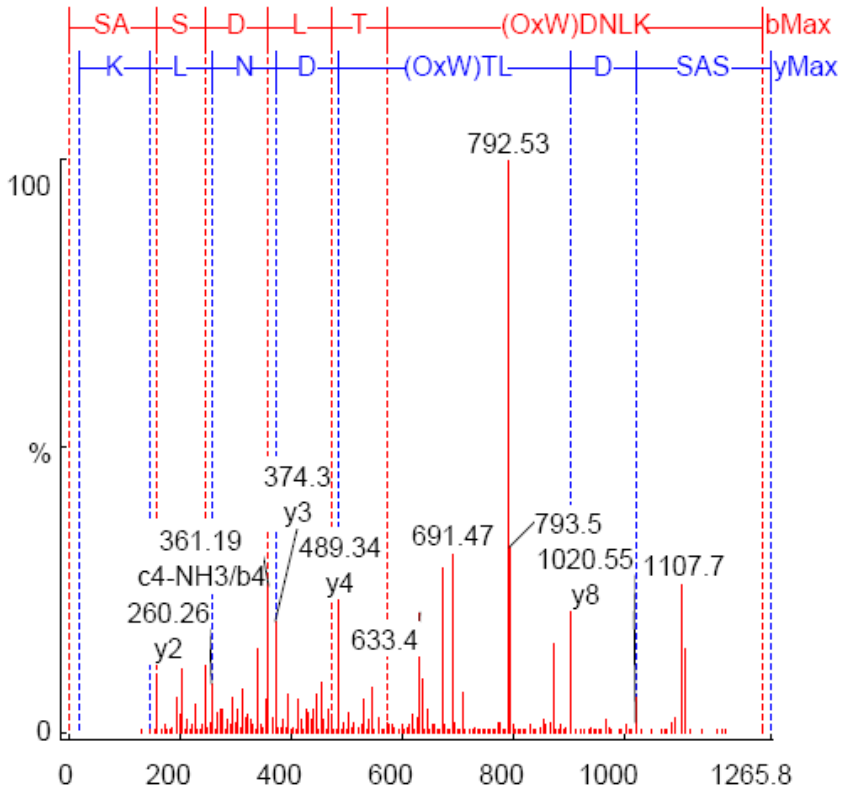


Fig. 9: MS/MS spectrum of *m/z* 633.4001 from *in vitro* sample DT-4, annotated by PEAKS after database matching against transferrin (Accession gi|15021381; 99% confidence).

Overall, The highest scoring was recorded for the samples exposed to both oxidative stress and iron incorporation.

No significant differences were observed between native and denatured protein samples. Mapping of the oxidized residues onto the 3-D structure of Horse Spleen Ferritin showed them to be located on the inner face of each sub-unit, facing toward the ferritin core where iron is normally stored, as shown in Fig. 10, 11 and 12. Furthermore, methionine was oxidized to a greater extent than tryptophan or histidine yielding the highest scoring of modified peptides for both ferritin and transferrin in both *in vivo* and *in vitro* experiments. We suggest that the use of catalytic amounts of iron, rather low concentrations of hydrogen peroxide are sufficient to oxidize methionine. In the first portion of our *in vitro* work, we obtained an abundance of modified methionine residues in the presence of EDTA (iron chelator). In the second portion of our *in vitro* work, however, we obtained even higher scoring oxidatively modified methionine residues in the absence of EDTA.

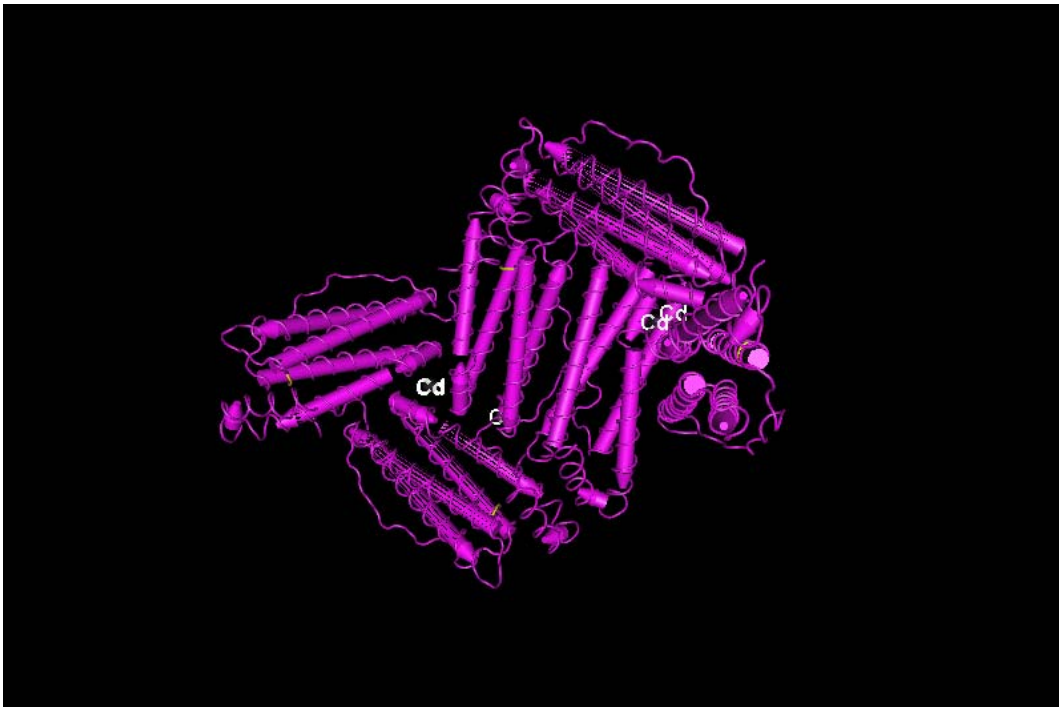


Fig.10: 3-Dimentional structure of Horse Spleen Ferritin Molecule. Oxidized Met residues (yellow) are located on the inner face of each sub-unit.

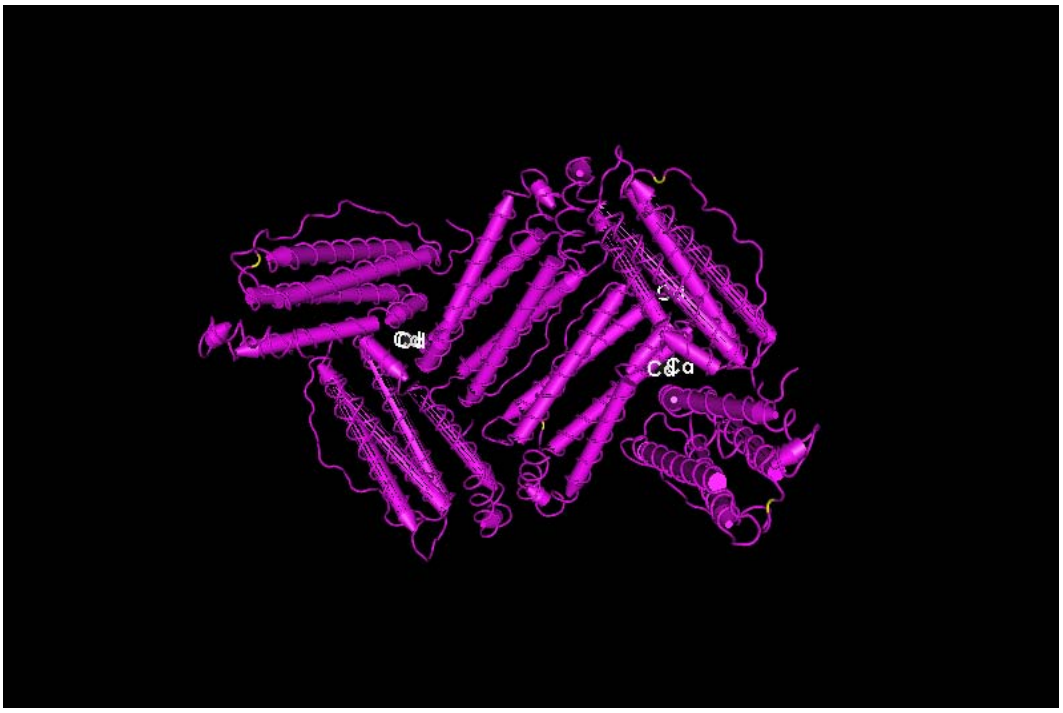


Fig.11: 3-Dimentional structure of Horse Spleen Ferritin Molecule. Oxidized Trp residues (yellow) are located on the inner face of each sub-unit.

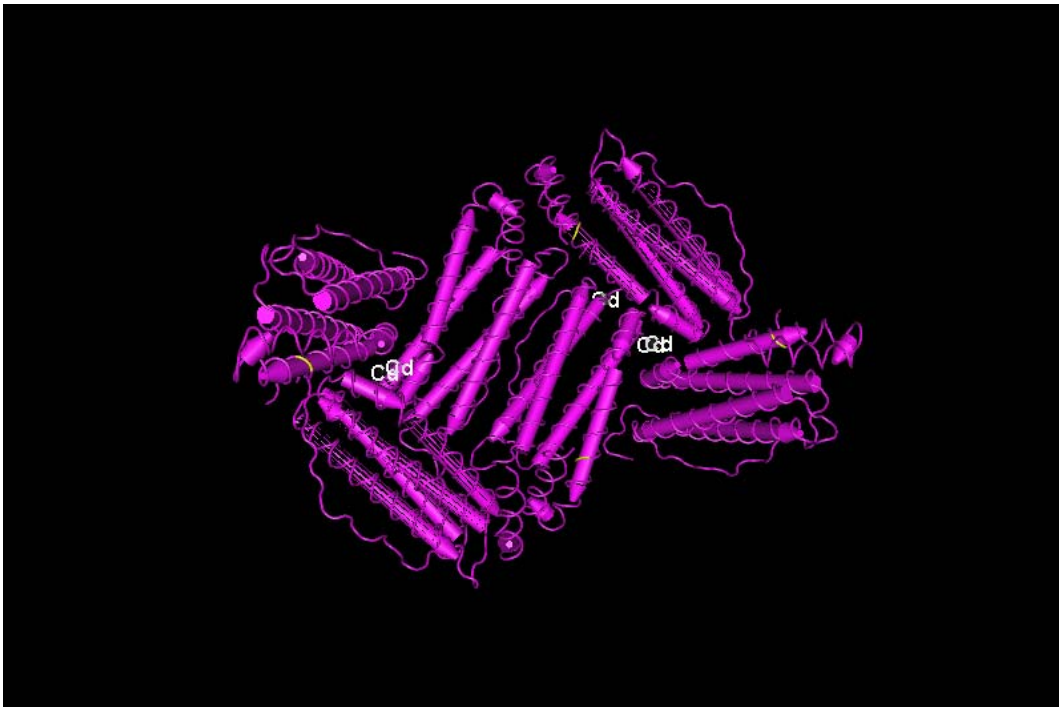


Fig.12: 3-Dimentional structure of Horse Spleen Ferritin Molecule. Oxidized His residues (yellow) are located on the inner face of each sub-unit.

6.4 Oxidized peptides identified during *in vitro* experiments and selected for inclusion and targeted Q-TOF LC-MS/MS analysis of biological samples

6.4.1 Transferrin

Inclusion List (used for targeted LC-MS/MS analysis of HH patient samples)

m/z	charge	sequence
430.2325	(2+)	NPDP(OOW)AK
526.9611	(3+)	KSASDLT(OxW)DNLKGK
532.0118	(3+)	TAGWNIP(OxM)GLLYNK
544.8204	(4+)	DEYKDC(OxH)LAQVPSETVVAR
599.8873	(2+)	(OOH)QTVPQNTGGK
606.3192	(2+)	DSGFQ(OxM)NQLR
633.3508	(2+)	SASDLT(OxW)DNLK
641.3419	(2+)	SASDLT(OOW)DNLK
642.3898	(3+)	EFQLFSSP(OOH)GKDLLFK
666.6204	(4+)	RKPVDEYKDC(OxH)LAQVPSHTVVAR
698.1269	(4+)	S(OxM)GGKEDLIWELLNQAQEHFGKDK
747.9221	(2+)	(OxM)YLGYEYVTAIR
762.4345	(3+)	RSAG(OxW)NIPIGLLYCDLPEPR
781.4538	(3+)	GRSAG(OxW)NIPIGLLYCDLPEPR
797.3975	(2+)	TAGWNIP(OxM)GLLYNK
805.4689	(2+)	TAG(OxW)NIP(OxM)GLLYNK
827.6469	(4+)	VK(OxH)STIFENLANKADRDNYELLCLDNTR
846.7921	(3+)	ALFQDLQKPSQDEWGTTLDA(OxM)K
855.2252	(4+)	RALFQDLQKPSQDE(OxW)GTTLDAMKAAIVLEK
1043.6183	(2+)	EDLI(OxW)ELLNQAQEHFGK

6.4.2 Ferritin

Inclusion List (used for targeted LC-MS/MS analysis of HH patient samples)

charge	m/z	sequence
400.9225	(3+)	(OxM)GDHLTNIQR
560.306	(3+)	RDDVALEGVC(OxH)FFR
600.8906	(2+)	(OxM)GDHLTNIQR
616.8919	(2+)	(OxM)GD(OOH)LTNIQR
846.7748	(3+)	ALFQDLQKPSQDEWGTTLDA(OxM)K
855.2383	(4+)	RALFQDLQKPSQDEWGTTLDA(OxM)KAAIVLEK
1111.9487	(3+)	LIKK(OOM)GD(OxH)LTNIQRLVGSQAGLGEYLFER

6.5 1-D SDS-PAGE for plasma samples of patients of HH

As described earlier, immunoprecipitants of plasma samples from patients with HH were run on SDS-PAGE. A molecular weight marker (MWT) was run in parallel with the plasma samples, shown at right in Fig.13. This figure also shows the transferrin bands for the samples P4A, P2 and P2A from right to left, respectively. Transferrin bands were visualized by Commassie stain at the molecular mass of 78 kDa. The appropriately sized bands were then excised from the gel, destained and digested with a proteolytic enzyme (trypsin) to generate discrete peptide fragments for which molecular masses were determined. The protein digest was analyzed by Q-TOF LC-MS/MS, which confirmed the identity of transferrin and indicated the oxidative modifications of the amino acids methionine and tryptophan for samples P2 and P2A. Sample P4A did not show any oxidative modification for transferrin. This will be discussed in more detail later.

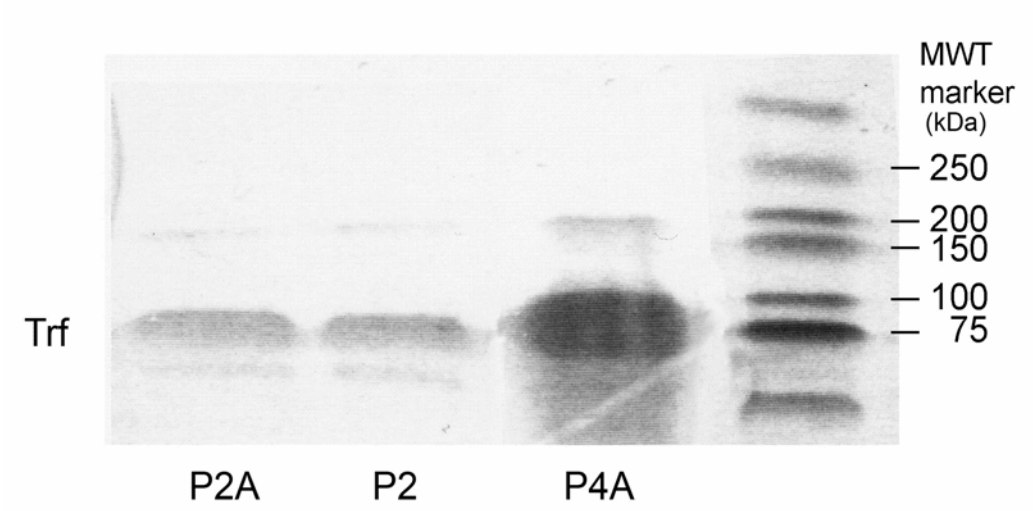


Fig.13: 1-D SDS-PAGE shows transferrin bands (Trf), visualized by Commasie stain at the molecular mass of 78 kDa from immunoprecipitants of plasma samples from patients with HH. The protein was loaded onto a 10% gel, stained by Commassie, destained and digested into peptide fragments by trypsin.

6.6 Summary of PEAKS database search results using data from targeted LC-MS/ MS analysis of in previous in vitro experiments of oxidized peptides

Transferrin was identified in 4 of the 7 samples (P2, P2A, P4 and P6), either through matching of target oxidized peptides identified during the *in vitro* experiments, or matching of unmodified peptides whose m/z values were sufficiently close to those of the target oxidized peptides (in the inclusion list) to be selected for MS/MS (the other four samples matched only albumin or immunoglobulin). Results for samples P2, P2A, P4A and P6 showed transferrin as the top hit. Results for the other samples indicate that the top scoring proteins were neither transferrin nor ferritin, which were not detected in these samples.

Peptides highlighted in red are target peptides identified in *in vitro* experiments. Of the four samples in which transferrin was found, only two (P2 and P2A) matched to transferrin using target oxidized peptides identified in the earlier in vitro experiments. Conducting database searches allowed for identification of addition of O or OO to M, H or W (as well as modification of W to kynurenine or 3-hydroxy-kynurenine) using the acquired peptide MS/MS spectra (i.e. those corresponding to the intended, target peptides in the inclusion list, or anything close enough in m/z to be selected for MS/MS). As a result, some peptides were matched to transferrin on the basis of one or more of these modifications, even though such peptides were not found in the *in vitro* experiments. However, the confidence with which these individual peptides were matched to transferrin was low, while identification of transferrin was based mainly on high-confidence matches from target (i.e. selected) oxidized peptides and/or unmodified peptides that happened to be selected for MS/MS. Consequently, only the three target oxidized peptides that matched transferrin were

considered 'real', confirming one oxidized tryptophan and two oxidized methionine residues in transferrin, as will be detailed later.

6.6.1 Sample P2

Table12 shows m/z, charges, and scores of different modified and unmodified peptides of protein digest of plasma Sample P2 of a patient with HH.

Mass: 63407.215 **Score:** 27.27% **Coverage:** 14.19%

Mz	Charge	Mr(calc)	Start	End	Score	Peptide
403.1744	3	1206.563	533	541	18.23%	L(OOH)DRNTYEK
544.9492	3	1631.8201	132	146	40.08%	DCHLAQVPSHTVVAR
633.15735	2	1264.5935	327	337	7.83%	SASDLT(OxW)DNLK
762.3158	2	1522.7415	171	183	4.57%	SKEFQLFSSP(OOH)GK
796.78906	2	1591.7156	389	403	2.83%	LCMGSGLNLCEPNNK
797.5013	2	1592.802	349	362	83.19%	TAGWNIP(OxM)GLLYNK
853.9678	2	1705.8999	326	340	2.45%	KSASDLT(OxW)DNLKGKK

MS/MS analysis of the oxidatively modified peptide, SASDLT(OxW)DNLK **(***m/z* 633. 15735) shows a score of 7.83% for OxW with a single O atom (See Fig.15). However, the analysis of the same sample for the oxidatively modified peptide, KSASDLT(OxW) DNLKGKK (m/*z* 853.9678) revealed a score of 2.45% with a single O atom.

MS/MS analysis of the same plasma sample for oxidatively modified peptide, TAGWNIP(OxM)GLLYNK, (*m/z* 797.5013) shows a significant score of 83.19% for OxM with a single O atom (See Fig. 14).

MS/MS analysis of the oxidatively modified peptides, L(OOH)DRNTYEK (*m/z* 403.1744) and SKEFQLFSSP(OOH)GK (*m/z* 762.3158) show scores of 18.23% and 4.57% for OOH with two O atoms .

Figure 14 shows the annotated MS/MS spectra of *m/z* (633.17535) from the *in vivo* sample in which two y-type ions are shown (y2, y3). y-type ions retain the C-terminal residues. Two z-

type ions (z2, z5-type) are also shown which retain the C-terminal residue as well. However z-type ions are weakly represented in this figure due to the fact that they only show abundance in high energy CID experiments. D(OxW)TLDSAS and LT(OxW)DNLK represent the theoretical partial sequences developed from C-terminal (y-type) and N-terminal (b-type) respectively.

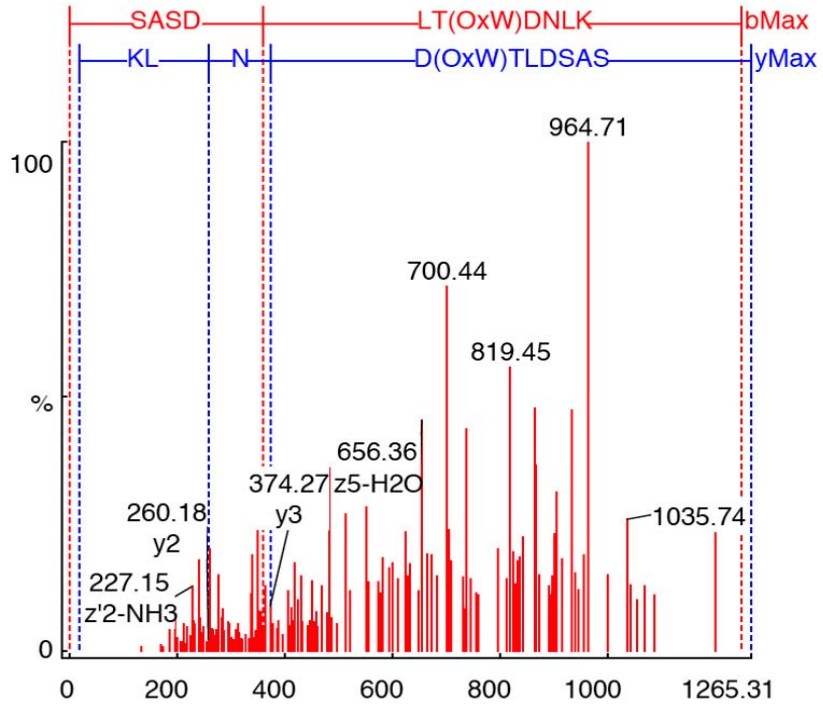


Fig.14: Annotated MS/MS spectrum for the peptide SASDLT(OxW)DNLK, *m/z* 633.17535, showing singly charged y-type ion. b-type ion are not annotated

Figure 15 shows the annotated MS/MS spectra of *m/z* (797.5013) from the *in vivo* sample P2. four y-type ions are shown (y2, y3, y4, y5). One b-type ion (b3 -type) is also shown which retains the N-terminal residue. GI(OxM)PINWGAT and IP(OxM)GLLYNK represent the theoretical sequences developed from C-terminal (y-type) and N-terminal (b-type) ions, respectively.

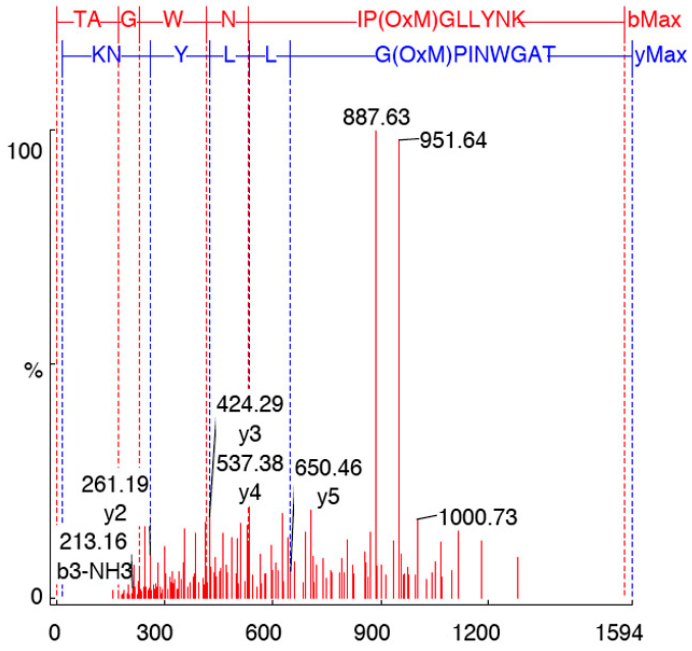


Fig. 15: Annotated MS/MS spectrum for the peptide TAGWNIP(OxM)GLLYNK (*m/z* 797.5013) showing singly charged ion y3.

6.6.2 Sample P2A

Table 13 shows m/z, charges, and scores of different modified and unmodified peptides of protein digest of plasma **Sample P2A** of a patient with HH.

Mass: 131854.62 **Score:** 22.28% **Coverage:** 5.12%

Mz	Charge	Mr(calc)	Start	End	Score	Peptide
531.30615	2	1060.5164	1197	1205	17.12%	VFGWPQCPK
544.98987	3	1631.8201	694	708	4.38%	DCHLAQVPSHTVVAR
431.4575	3	1291.5503	399	409	5.84%	WCAVSE(OOH)EATK
606.30426	2	1210.54	558	567	94.33%	DSGFQ(OxM)NQLR
1043.561	2	2084.996	146	162	4.32%	RLPGV(OHKyW)(OOW)CAVGH(OOH)ERAK

MS/MS analysis of the oxidatively modified peptide DSGFQ(OxM)NQLR (m/z 606.30426) shows a significantly high score of 94.33% for OxM with a single O atom (See Fig.16). However, the analysis of the theoretical peptide modified by His (OOH) (m/z 431.4575) revealed a non significant low score of 5.84% with 2 O atoms. MS/MS analysis of oxidatively modified peptide RLPGV(OHKyW)(OOW)CAVGH(OOH)ERAK (m/z 1043.561) revealed a non significant score of Trp oxidation products, 3-hydroxykynurenine (OHKyW) and OOW. As shown in Fig.16, OxM showed a strong y-type ion series y1, y2, y4, y6 and y7. However, one a-type ion is also shown (a2-) which retains the N-terminal residue. The presence of these ions in the spectrum increases the score of the sequence containing OxM (94.33%) and enables confident assignment of both the site and the type of modification. The relatively high signal to noise (S/N), apparent in Fig.16, adds to more confidence in identifying the site of modification.

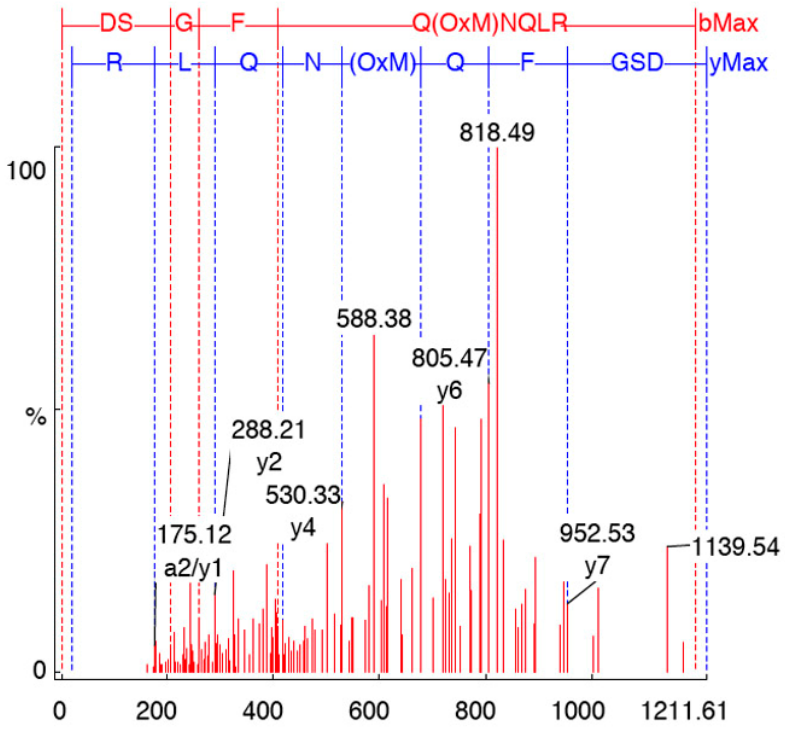


Fig. 16: Annotated MS/MS spectrum for the peptide DSGFQ(OxM)NQLR (*m/z* 606.30426) showing singly charged y-ion series .

6.6.3 Sample P4A

Table 14 shows m/z, charges, score of the minimally modified peptide, CLVDLN(KyW)NNLEGK of protein digest of plasma **Sample P4A** of a patient with HH

Mass: 1.0068246E7 **Score:** 5.18% **Coverage:** 2.06%

Mz	Charge	Mr(calc)	Start	End	Score	Peptide
761.42236	2	1520.7292	588	600	26.61%	CLVDLN(KyW)NNLEGK

MS/MS analysis of the peptide CLVDLN(KyW)NNLEGK (m/z 761.42236), shows a relatively low score of 26.61% for KyW (See Fig.17).

Figure 17 shows the annotated MS/MS spectra of *m/z* 761.42236 from the *in vivo* sample P4A. Seven y-type ions are assigned (y2, y4, y5, y7, y8, y9, y12).

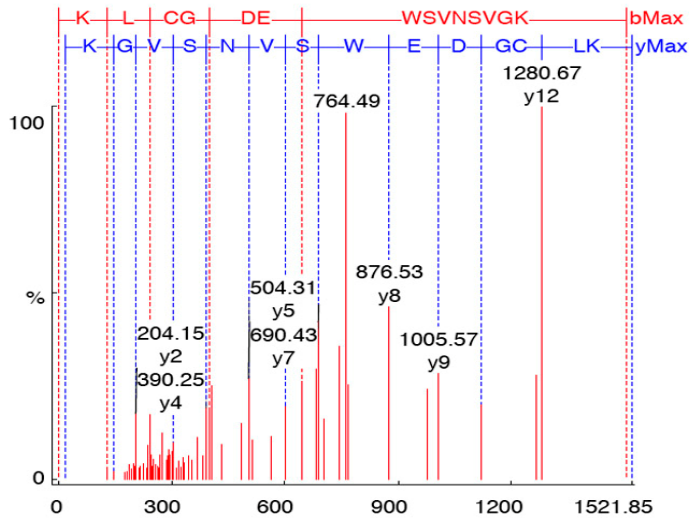


Fig. 17: Annotated MS/MS spectrum for the peptide CLVDLN(KyW)NNLEGK *m/z* 761.4223 with singly charged y-type ions. b-type ions are not shown.

Figure 18 shows the annotated MS/MS of *m/z* (761.4239) from tissue sample P4A (the same above sample but different peptide.

Seven y-type ions are shown (y2, y4, y5, y7, y8, y9, y12). y-type ions retain the C- terminal residues.

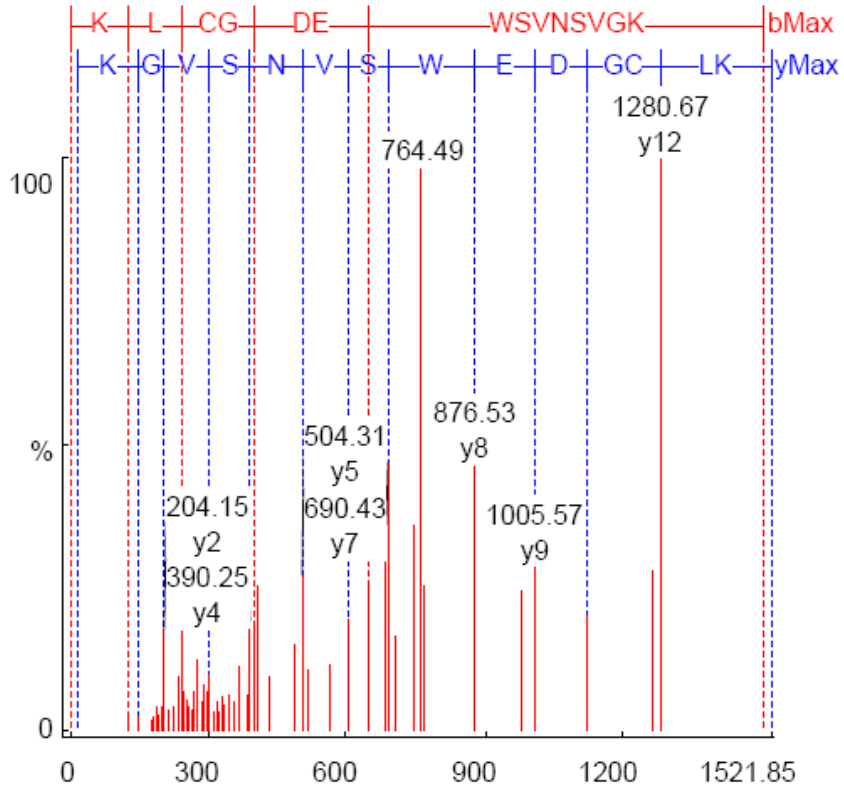


Fig. 18: MS/MS spectrum of *m/z* 761.4239 from tissue sample P4A, annotated by PEAKS after database matching against transferrin (Accession gi|62897069; 88.2% confidence).

6.6.4 Sample P6

Table 15 shows m/z, charges, score of the minimally modified peptides, DECMVKWCAIG(OxH)QER and TSDANIN(KyW)NNLK of protein digest of plasma **Sample P6** of a patient with HH.

Mass: 77753.2 **Score:** 7.54% **Coverage:** 3.84%

Mz	Charge	Mr(calc)	Start	End	Score	Peptide
607.53143	3	1819.7803	360	374	37.04%	DECMVKWCAIG(OxH)QER
697.3981	2	1392.6633	457	468	2.16%	TSDANIN(KyW)NNLK

The MS/MS analysis of the peptides DECMVKWCAIG(OxH)QER, m/z (607.5314) and TSDANIN(KyW)NNLK m/z (697.3981) show a relatively low score of 37% for OxH and 2.16% for KyW respectively.

6.6.5 List of in vitro samples that have most fragment ion peaks in common with oxidized peptide MS/MS spectra from samples of patients with HH

1) Peptide DSGFQ(OxM)NQLR (m/z 606.3042) found in sample P2A (see Fig. 17, page 106), the best match was the (m/z 606.3192) MS/MS spectrum from the *in vitro* sample 002 (see Fig. 7, page 78), which matched transferrin with 73.2% confidence. Peaks were observed in both spectra are m/z 175.1 (a2/y1), 288.2 (y2), 530.3 (y4), 805.5 (y6) and 952.5 (y7).

2) Peptide SASDLT(OxW)DNLK (m/z 633.1517) found in sample P2 (see Fig. 15, page 101), the best match was the m/z (633.4001) MS/MS spectrum from the *in vitro* sample DT4 {denatured transferrin treated with 20 uM hydrogen peroxide and 2 equivalents of FeNAc3}, (Fig.10, page 89), which matched transferrin with 99% confidence. Peaks were observed in both spectra are m/z 260.2 (y2), 374.3 (y3) and 633.4 (the doubly-charged precursor ion itself).

3) Peptide TAGWNIP(OxM)GLLYNK (m/z 797.5013) also found in sample P2 (see Fig.16, page 103), the best match was the m/z (797.5536) MS/MS spectrum from the *in vitro* sample NT4 {native transferrin treated with 20 uM hydrogen peroxide and 2 equivalents of FeNAc}, (Fig. 9, page 86), which matched transferrin with 65.3% confidence. Peaks observed in both spectra are m/z 261.2 (y2), 424.3 (y3), 887.6 (unassigned), 951.6 (y8) and 797.6 (the doubly-charged precursor ion itself).

4) A MS/MS spectrum for an unmodified peptide KLCGDEWSVNSVGK (m/z 761.4224, 2+ charge) that was detected in a real sample (P4) because it occurred to have a m/z ratio close to one of the target modified (oxidized) peptides, CLVDLN(KyW)NNLEGK, found in

our *in vitro* samples, and which matches transferrin with good confidence (88%), confirming that this protein is present.

6.7 Detection of protein carbonyls in plasma samples of patients with HH

As shown in Fig. 19 (B), compared to the controls, samples 2, 3, 4, 5 recorded a significantly high content of protein carbonyls (1-7 times) which correlated with the relative levels of serum ferritin and transferrin saturation regardless of whether the patients were homozygous or heterozygous to C282Y or H63D. Sample 2 recorded the highest carbonyl content among all (80.3 ± 44.8 pmol/μL). This sample was from a patient with active hepatitis due to HCV infection, as well as high serum ferritin, 1517 ng/L (normal: 20–250 ng/L) and high serum transferrin saturation, 93% (normal: 17 – 50%) (Table 3, Page 50). The genotypic testing of this patient revealed one copy of H63D. The lowest carbonyl content was from a patient with serum ferritin of 182 ng/L and serum transferrin saturation of 54%. This patient reported a carbonyl content of 15.0 ± 20.5 pmol/μL. The genotypic testing of this patient revealed two copies of C282Y (Table 3, Page 50). The average from our controls (n = 6) recorded a carbonyl content of 10.03 ± 4.2 pmol/μL plotted against the average from patients with HH (n = 6) which had a carbonyl content of 35.5 ± 23.9 pmol/μL (Fig. 19 A). These findings compared well with those of Houglum et al. (1997). They tested 15 subjects for each group of control as well as untreated and treated patients with genetic hemochromatosis. According to Houglum et al., genetic hemochromatosis patients had increased transferrin saturation and serum ferritin levels prior to treatment compared to control and treated patients. Their data and ours showed a significant positive correlation between the level of carbonyl content and serum ferritin concentration as shown in Fig. 20 which shows a linear relationship between ferritin content and carbonyl content of plasma samples from controls and patients with HH.

6.7.1 Statistical Analysis

Unpaired *t* test results:

P value and statistical significance**:** The two-tailed P value equals 0.0278 (P< 0.05), by conventional criteria, this difference is considered to be statistically significant.

Confidence interval:

The mean of Controls minus HH Patients equals -25.4700

95% confidence interval of this difference: From -47.5434 to -3.3966

Intermediate values used in calculations:

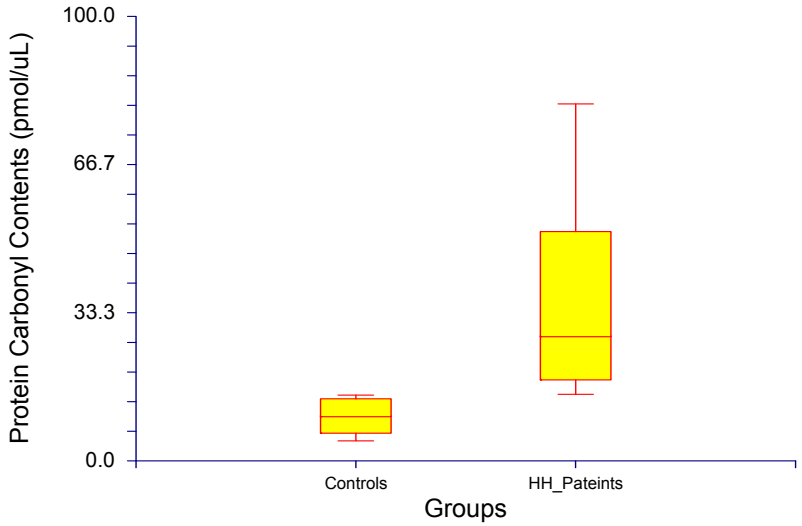
t = 2.5710

df = 10

standard error of difference = 9.907

Group	Controls	HH Patients
Mean	10.0300	35.5000
SD	4.2000	23.9000
SEM	1.7146	9.7571
N	6	6

(A)



(B)

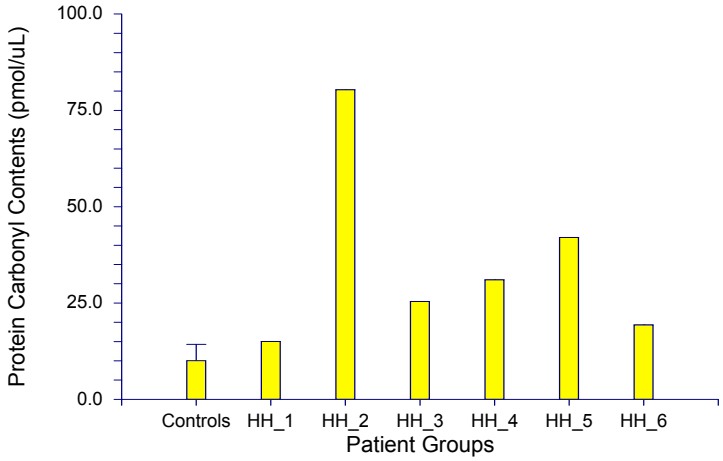


Fig.19 (A) and (B): Carbonyl groups, an index of oxidative stress, are increased in HH Patients. (A) Box Plots of Carbonyl groups controls (n = 6; 10.03 ± 4.2 pmol/μL) and HH patients (n = 6; 35.5 ± 23.9 pmol/μL). (B) Standard Deviation Bar Graph of Controls and Individual HH Patients Samples.

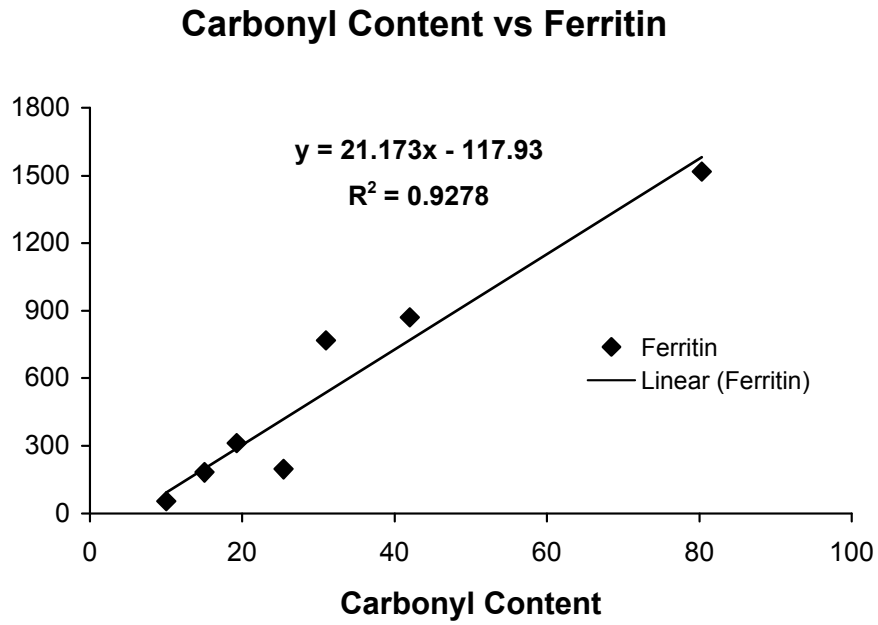


Fig. 20. Linear relationship between carbonyl content and ferritin in plasma samples from patients with HH and controls.

7.0 Discussion

The detection of the oxidative modification of proteins has been used as a marker of oxidative stress. Oxidative stress can occur when ROS production is accelerated or when the mechanisms involved in maintaining the normal reductive cellular milieu are impaired (either by depletion of antioxidants or mutations affecting antioxidant defence enzymes such as Cu/ Zn-SOD). Increased production of ROS is thought to occur more frequently than diminished antioxidant defence (Dalle-Donne, I. et al. 2003). It should be emphasized that the detection of more than one marker for oxidative stress is strongly recommended to avoid the misleading results that might be given by depending on only one marker. In our experiments, we detected more than one marker to confirm oxidative injury that may lead to disturbance of iron homeostasis in untreated patients with hereditary hemochromatosis.

We were able to detect oxidative modifications in different amino acid residues such as tryptophan, methionine and histidine *in vitro* by inducing oxidative stress and iron overload in commercial horse spleen ferritin and human serum transferrin (Sigma). The proteins were subjected to tryptic digestion prior to analysis by Q-TOF LC-MS/MS. The modified peptides were targeted for possible identification of oxidative modifications in plasma samples of patients of hereditary hemochromatosis.

7.1 Protein Carbonylation and Lipid Peroxidation as Biomarkers of Oxidative Stress

Protein carbonyl content is the most general and well-used biomarker of severe oxidative protein damage. Protein carbonyls have a major advantage over the lipid peroxidation products as markers of oxidative stress because of their stability and their early and prolonged circulation in the blood. In our experiments, we used the detection of protein carbonyls as a preliminary and general procedure to indicate if oxidative stress has a role in

the pathophysiology of hereditary hemochromatosis. For this purpose, 2, 4-dinitrophenylhydrazine (DNPH) was used to detect protein carbonyls in the plasma samples of untreated patients of hereditary hemochromatosis. This technique is based on the reaction between protein carbonyls and DNPH resulting in the formation of a Schiff base to produce the corresponding hydrazone, which can be analyzed spectrophotometrically. A significantly high content of protein carbonyls (1-7 times), compared to the control, were detected. These findings correlate positively with the relative levels of serum ferritin. One of our samples recorded a particularly high carbonyl content. This sample was from a patient with active hepatitis due to HCV infection as well as high serum ferritin, 1517 ng/L (normal : 20 - 250 ng/L) and high serum transferrin saturation, 93% (Normal : 17 - 50%). This finding supported the suggestions of Koike, K. et al. (2006) that oxidative stress is involved in the pathogenesis of hepatitis and hepatocellular carcinoma. However, the genotypic testing of this patient revealed one copy of H63D. Piperno et al. (1992) studied the presence of antibodies to hepatitis B and C viruses, serum ALT concentrations, hepatic histology, and total body iron stores (assessed by phlebotomy) in 78 Italian patients with hemochromatosis. We also reported another patient who recorded a relatively low carbonyl content despite the fact that he was homozygous for C282Y but serum levels of ferritin and transferrin saturation were low. There was no significant difference reported between the carbonyl contents of homozygous or heterozygous individuals for C282Y and H63D which supports the suggestion of the involvement of oxidative stress in the pathogenesis of hereditary hemochromatosis due to the oxidative modifications of ferritin and transferrin regardless of whether the patients were homozygous or heterozygous for the C282Y and H63D mutations (unpublished observation).The positive correlation between high plasma carbonyl content and high plasma ferritin suggested that the pool of catalytic iron increases in parallel with the

hepatic iron and may be linked to enhanced lipid peroxidation (Houglum, K.et al 1997). This positive correlation still does not explain any direct links between *HFE* and ferritin metabolism but it is possible that the abnormal protein synthesized as a consequence of mutated *HFE* gene directly or indirectly leads to disturbances in ferritin metabolism or iron loading. In HH, elevated plasma ferritin occurs in the absence of architectural damage to the liver, suggesting that the elevated plasma ferritin results, most likely, from enhanced secretion of ferritin rather than tissue damage. At this point, the plasma proteins that are oxidatively modified in untreated patients with HH, as measured by carbonyl assay, have not yet been characterized since the elevation of carbonyl levels may be due to the oxidation of any other plasma protein rather than transferrin. We characterized the oxidatively modified plasma protein (transferrin) by using an antibody to isolate it for tryptic digestion and Q-TOF LC-MS/MS analysis.

Houglum et al. (1990) have indicated that plasma proteins synthesized by hepatocytes such as transferrin, are modified by MDA- and 4-HNE-lysine adducts, decomposition products of lipid peroxidation in a rat model of iron overload. As well, they have detected MDA-lysine adducts and autofluorescence in hepatocytes of iron-overloaded rats. In support of a hepatocellular origin for aldehyde-protein adducts is the finding that levels of 4-HNE and MDA-protein adducts in the blood circulation of animals are stable despite the removal of the iron from the diet (Houglum et al. 1990). Furthermore, Houglum et al. could not detect the presence of an aldehyde-transferrin adduct, as transferrin did not correspond with MDA and 4-HNE in the plasma samples of iron-overloaded rats. Shacter et al. (1995) did not suggest transferrin as a major plasma protein target for oxidative modifications. Free or catalytic iron increases in the livers of rats overloaded with iron and it is thought that this iron pool, and not iron sequestered in ferritin or hemosiderin, promotes lipid peroxidation (Britton et al. 1990).

Surprisingly, Houglum et al. (1997) could not detect MDA-protein adducts in plasma proteins of plasma samples of patients of HH using the same Western blot analysis technique used with plasma samples or iron-overloaded rats. They indicated that the cause for this might be the insidious rate of iron accumulation in the liver of HH patients which contrasts with the rapid iron loading in the animal model.

Carbonyl iron (a highly purified form of elemental iron) has been used by Bacon and colleagues (1983) to evaluate the role of chronic iron overload in promoting lipid peroxidation *in vivo* by examining subcellular fractions of liver from chronically iron-loaded rats for the presence of lipid conjugated dienes, intermediates in the peroxidative degradation of polyunsaturated lipid membranes. They supplemented the diet of Sprague-Dawley male rats with 2.5% (wt/wt) carbonyl iron. This feeding regimen predominantly produced iron loading of hepatocytes in a peripheral distribution, similar to that of hemochromatosis. Significant quantities of iron were deposited after, as early as, two weeks in rats fed on the carbonyl-iron diet. Iron continued to accumulate in hepatocytes for three months until stores of iron in hepatocytes were 40-90 times of those of control animals with the development of signs of hepatic necrosis. After eight months, Kupffer cells and macrophages were heavily invaded with iron deposits. Twelve months were quite enough to ensure hepatic fibrosis and necrosis reproducing an experimental model of the pathophysiology of hemochromatosis. We suggest that Bacon et al. may have designed a model for hemochromatosis based on the fact that iron overload leads to hepatic injury due to oxidative stress. This may not be an ideal model for HH because some other factors, such as the levels of plasma ferritin and transferrin saturation, should be considered to avoid confusion with any other iron overload disorder. However, this model has been described before even the discovery of *HFE* gene in 1996. Many other groups have used this model to demonstrate the hepatotoxic effects of

excess iron on peroxidation of organelle membranes, organelle function including lysosomal fragility, impairment of mitochondrial oxidative metabolism, abnormalities in calcium transport , reduction in the levels of cytochromes a, b, c and cytochrome C oxidase activity and disruption of metabolic functions of other subcellular organelles (Britton, et al. 1990; Houglum,et al. 1990; Fletcher, et al. 1989 and Myers et al. 1991). We have confirmed the presence of protein carbonyls in real samples (plasma samples of untreated patients with HH)while considering ferritin and transferrin saturation levels as well.

7.2 Why Hydrogen Peroxide?

In our *in vitro* experiments we have induced oxidative stress in commercial protein standards (Horse Spleen Ferritin and Human Serum Transferrin) by using hydrogen peroxide, which has been extensively used as an oxidant. As known, most of the oxidative damage in tissues require the participation of catalytically active iron (Rudeck, M. et al. 2000). Free iron can catalyze the decomposition of hydroperoxides, including hydrogen peroxide, resulting in the formation of highly reactive substances that promotes lipid peroxidation and other oxidative damage reactions. An increase of this reactive iron is believed to be a pathophysiological factor in several diseases that are accompanied by oxidative damage. To prevent this oxidative damage, a system has evolved to limit the concentration of reactive iron but also to provide iron for biochemical synthesis. Ferritin, as an intracellular protein, binds iron in a catalytically inactive form; accordingly, oxidative reactions cannot be promoted by this iron. Because the main iron pool in most cells is located within the ferritin, it is important to know under what circumstances, if any, this pool may be a source of iron. Ferritin has been shown to release iron on contact with activated microglia and neutrophils by a superoxide-dependent mechanism.

Oxidative damage to ferritin that results in a physiological malfunction e.g., the release of iron,might be accompanied by damage to the apoferritin molecule. Protein oxidation in mammalian cells is generally accompanied by the degradation of oxidized protein molecules. However, hydrogen peroxide-induced protein oxidation enhances the proteolytic susceptibility of the damaged protein.

In our *in vitro* experiments, we have detected oxidative damage to ferritin and transferrin molecules following exposure to two different concentrations of hydrogen peroxide (10 and 20 μM). This oxidative damage leads to the modification of both proteins. This was confirmed

by Q-TOF LC-MS/MS which detected a number of oxidatively modified peptide fragments containing oxidized methionine, histidine, and tryptophan that were identified using PEAKS software. Mapping of the oxidized Ferritin residues showed that the modified residues were located on the inner face of each sub-unit, the face directed toward the ferritin core where inert iron is normally stored.

Our findings partially correlate with those of Rudeck and co-workers who showed that the treatment of ferritin with hydrogen peroxide increases the oxidative damage and proteolytic susceptibility of the ferritin molecule dramatically, since iron is released before the maximal possible degradation of oxidized ferritin has occurred compared to other oxidants such as xanthine / xanthine oxidase and DEA-NO (Rudeck, et al. 2000). We used EDTA in our first set of experiments along with hydrogen peroxide as EDTA-Fe (III) complexes to effectively catalyze ·OH formation, as reported by Aust, S. et al. (1985). The rate of lipid peroxidation is dependent upon the ratio of chelate (EDTA) to ferric iron. A ratio of less than 1 maximizes the rate of lipid peroxidation (Aust et al. 1985). A ratio of 1:1 is still able to initiate lipid peroxidation ; however, a ratio above 1 causes inhibition of lipid peroxidation. The concentration of EDTA we have used in our experiments was 10 mM, which was chosen to stop the oxidation reaction at a specific point, prior to the dialysis step, and to enhance the autoxidation of the proteins. However, we did not detect protein carbonyls in our *in vitro* experiments using Q-TOF LC-MS/MS probably because of our low concentration of hydrogen peroxide (10, 20 μM) or the absence of derivatization with DNPH prior to the oxidation process (unpublished observation). Headlam, H. et al (2004) have indicated that reaction of BSA with hydrogen proxide alone over the range of 290-1450 μM gave only low levels of released carbonyls, and the yield of each of these materials did not increase significantly with increasing oxidant concentration (lowest yields compared to HOCl, γ radiolysis and other

oxidants). However, the addition of Fe (II) (± EDTA) at a fixed concentration to hydrogen peroxide (290 μM) gave a significant increase in the levels of all released carbonyls. In contrast, increasing concentrations of Fe (II)-EDTA (in the absence of hydrogen peroxide) gave significant dose-dependent increases in the yields of released carbonyl compounds (Headlam, H. 2004).

7.3 Autoxidation and Iron Incorporation in Ferritin Molecules

The goal of the first part of our second set of *in vitro* experiments was to incorporate iron within horse spleen ferritin by using ferrous ammonium sulfate with ferritin along with two different concentrations of hydrogen peroxide (10, 20 μM) without the involvement of an iron chelator (EDTA). The incorporation of iron into the ferritin core using ferrous ammonium sulfate was dependent on the buffers used, with HEPES and Tris being the most effective, as well as pH of the buffers. As indicated by Silva, D et al. (1992) at pH 7.0, the initial rate of iron incorporation was about 0.1 μM/min. When the assay was repeated at pH 6.0, the rate of incorporation dropped to about 0.01μM/min. At neutral pH, Fe (II) is susceptible to hydrolysis and autoxidation due to the deprotonation of iron-bound water molecules. As the pH decreased, the stability of Fe (II) increased.

The presence or absence of iron chelator is also an important factor that might affect iron incorporation within the ferritin core. However, the presence of a strong iron chelator such as EDTA or Diethylenetriaminepentaacetic Acid (DPTA) will stabilize Fe (II) preventing or lessening the iron incorporation into the ferritin core which will lead to slowing the rate of autoxidation due to iron overload.

We induced oxidative stress using hydrogen peroxide in 50 mM HEPES buffer, pH 7.0 without the involvement of EDTA in these preparations. Silva, et al. (1992) have reported that incubation of iron-ferritin complex with EDTA for 4 hours was sufficient to remove up to 30% of the iron from ferritin loaded with ferrous ammonium sulfate. HEPES buffer, which is a poor chelator of iron, allows the greatest oxidation of ferrous iron since it does not compete well, either with protein for metal binding or with water for coordination to free sites on the metal. However, the incorporation appeared to be related to the rate of autoxidation (De Silva et al. 1992), i.e. the more iron incorporation, the highest rate of autoxidation obtained. The rapid

autoxidation of Fe (II) can give rise to the production of superoxide, hydrogen peroxide, and the hydroxyl radical via the iron-catalyzed Haber-Weiss reaction. These species are capable of altering biological molecules such as ferritin via modification of amino acid residues (Stadtmen, E. et al. 1991), and may result in the loss of integrity of the protein. It is known that the oxidation of proteins results in preferential proteolysis *in vivo*. The availability of iron and hydrogen peroxide is fundamental to these metal-catalyzed oxidation processes. The production of hydroxyl radical during the non-enzymatic deposition of iron into ferritin has already been demonstrated, and it was proposed that this was confined to the interior of the molecule (data not published) which undoubtedly causes localized damage to the protein. On the other hand, according to De Silva et al. (1992), the loading of apoferritin by ceruplasmin was not dependent on the autoxidation of iron, and amino acid analysis revealed no damage to the protein shell during iron incorporation in this system. This would account for the lack of amino acid oxidation observed during ceruplasmin-dependent loading of ferritin. Welch, K. et al. (2001) could not determine the effect of loading of iron into ferritin via ceruplasmin on the modification of tryptophan which was not the case in our experiments using ferrous ammonium sulfate as an iron source for incorporation within ferritin core. Welch et al. have demonstrated that loading iron into ferritin altered the 3-D conformation or tertiary structure of the protein such that new sites were accessible to trypsin. Many of the new recognition sites were close to cysteine residues. This suggests that the cysteine residues were oxidized and consequently the tertiary structure of the protein in the vicinity of the cysteine residues was altered. Many studies have shown that oxidation does alter the tertiary structure of the protein.

7.3.1 Ferritin oxidation and ·OH production

According to Grady, J. et al. 1989, ·OH produced from the iron-catalyzed Haber-Weiss

reaction is a by-product of core formation in ferritin. As described above, radical production appears to be confined to the interior of the ferritin molecule, where cellular components would be protected from the oxygen-mediated oxidative damage to the protein, a process that may contribute to the formation of hemosiderin from ferritin.

Hemosiderin is thought to be formed from the aggregation and the partial degradation of the ferritin protein shell by lysosomal enzymes. Hemosiderin peptides are derived from ferritin peptides and free radical catalyzed reactions are likely involved in the transformation. Hemosiderin is known to have smaller iron cores, and its protein shell is less complete than that of ferritin. While both proteins release iron and promote lipid peroxidation and hydroxyl radical formation, hemosiderin is consistently and significantly less effective than ferritin (Grady et al. 1989). It seems that transformation of ferritin into hemosiderin is a part of free radical mediated oxidation of the protein. Grady et al. have indicated that hydroxyl radicals were observed indirectly by using the spin-trapping reagent *N*-tert-butyl-α-phenylnitrone (PBN) at room temperature and directly by measuring ESR spectra of frozen molecules at 77 K, at which a PBN-R adduct is observed with both ferritin and apoferritin and not in the absence of protein. This suggests that the trapped species is derived from the protein itself and not simply from the iron core (Grady et al. 1989). However, diminution of the ESR signal by the use of known hydroxyl radical scavengers and enhancement of the signal by the addition of hydrogen proxide strongly suggested the involvement of hydroxyl radical in the formation of PBN-R adducts.

To summarise, the hydroxyl radicals are thought to be formed during iron mobilization from ferritin core by a strong chelator such as EDTA with the involvement of an oxidant such as hydrogen peroxide or via iron incorporation into ferritin core by ferrous ammonium sulfate or ceruplasmin.

7.4 Autoxidation and Iron Incorporation in Transferrin Molecules

Iron uptake by transferrin has been analyzed by a relatively small number of investigators. The goal of the second part of our second set of *in vitro* experiments was to incorporate iron within human serum transferrin by using ferric nitrilotriacetate along with two different concentrations of hydrogen peroxide (10, 20 μM) without the involvement of an iron chelator (EDTA). Twenty mM of sodium bicarbonate was added to transferrin to extract iron very rapidly from ferric nitrilotriacetate to yield an iron-loaded C-site complex which deprotonates and undergoes a fast change in conformation, followed by loss of two or three protons which is, in turn, followed by other slow changes of conformation leading to iron uptake by the N-site (Chahine, E. et al. 1996). The reaction of ferric salts with transferrin has been extensively studied by Bates, G. et al. (1973). They have reported that adding iron salts to buffered transferrin solutions in the neutral to alkaline pH range gives poor results. This was correlated with our preparations by adding nitrilotriacetic acid to ferric chloride at pH 2, raised gradually to 6, which provides a rapid and stable chelate-free ferric transferrin.

Bates, G. et al. (1973) have reported that titration of transferrin with ferric nitrilotriacetate gives a linear function and clear end point, while titration with ferric chloride results in a sigmoidal-shaped curve and no clear end point is reached. According to them, the spectral data indicate that only 5 to 25% of the iron becomes bound when 1 eq of ferric chloride is added to transferrin at neutral pH. The remaining sites of the protein are vacant and available for reaction with ferric nitrilotriacetate. In our methods, we have allowed one and two equivalents of ferric nitrilotriacetate to react with transferrin to bind the C-site iron load and total (C+N)-site iron load, respectively. As expected, we have detected much more modified methionine, tryptophan and histidine peptides when we used 2 equivalent of ferric nitrilotriacetate due to the occupation of C- and N–sites with iron rather than occupying only

the C-site using one equivalent of ferric nitrilotriacetate. As a consequence, iron uptake by transferrin leads to protein damage, which was detected oxidative modifications of methionine, histidine and tryptophan. However, no significant difference has been detected between the samples treated with low and high concentrations of hydrogen peroxide. The decomposition of hydrogen peroxide by ferric ions occurs but it is considerably slower than the Fenton's reaction.

Like the Fenton's reaction, it appears to be a redox chain reaction:

$$Fe\ (III) + H_2O_2 \Rightarrow Fe\ (II) + HO_2\cdot + H$$

This reaction, as indicated above, has an acidic pH optimum. At neutral pH, the reaction is extremely slow due to the tendency of ferric ions to hydrolyze. The reduction of ferric ions is critical as transferrin molecules have a weak affinity for ferrous ions that would facilitate its release, leading to transferrin autoxidation (Aust et al. 1985). It is speculated that at low pH, decreased stability of the Fe(III)-transferrin complex and the involvement of anions such as phosphate and bicarbonate are crucial for transferrin autoxidation. Iron incorporation has been reported *in vivo* by Bacon et al. (1983) after a single intraperitoneal injection of rats with ferric nitrilotriacetate (Fe-NTA) which gives rise to evenly distributed iron deposits over the hepatic lobules compared with the heavy iron deposits in hepatocytes due to dietary carbonyl iron. This is due, in part, to saturation of transferrin with subsequent increased endocytosis in a scenario which resembles a model for hereditary hemochromatosis. The low concentration of transferrin often found in the disease also appears to reflect the increased iron stores, and the concentration returns to normal after iron-depletion therapy. It seems unlikely that hemochromatosis is related to a primary defect in transferrin (Cartei, G. et al. 1975). Cartei et al. proposed a defect in the transferrin molecule but they did not define this defect. We suggest a possible defect in the transferrin molecule due to hereditary hemochromatosis.

This suggested defect is due to oxidative modification of transferrin yielding modified peptides of methionine, tryptophan and (data not published). Cartei et al. (1975) have demonstrated that venesection therapy causes the rise of plasma transferrin due to the removal of iron overload and not to venesection per se. Another possible defect in hemochromatosis is the decreased ability of reticuloendothelial cells to retain iron. The result of this defect is a return of greater amounts of iron from stores to plasma ferritin, increasing its iron saturation and thus causing more iron saturation and directing more iron into hepatocyte storage.

Furthermore, iron absorbed through the intestinal mucosal cells in the face of almost complete saturation of transferrin is taken up selectively by the hepatic parenchymal cells. Batey, R. et al. (1980) have confirmed the existence of a non-transferrin-bound iron (NTBI) fraction in the sera of patients with untreated or inadequately treated primary hemochromatosis. The high NTBI concentration in primary and secondary hemochromatosis, and in normal serum with an artificially elevated serum iron, suggest that the NTBI is a result of transferrin being fully saturated, thus preventing additional iron binding by serum transferrin, although this may not be the only explanation for the findings in primary hemochromatosis (Batey, et al. 1980).

Batey, et al. also have reported on four patients who, at diagnosis of primary hemochromatosis, had normal serum iron concentration and transferrin saturation and a raised NTBI level. This correlates with our record of having two untreated patients with normal plasma iron and transferrin saturation, although their genotypic tests confirmed C282Y and H63D mutations and having the clinical indicators of primary hemochromatosis. Batey's records and ours suggest that iron binding to transferrin may be altered because of some additional factor.

We suggest that this factor might be a defect in transferrin molecule that hinders the binding capacity of the transferrin receptor, and which is closely related to the oxidative transferrin modification confirmed by detection of protein carbonyls and modified methionine and tryptophan residues in plasma samples from these patients (data not published). Aruoma, O. et al. (1988) have confirmed the presence of low molecular weight iron in plasma from hemochromatosis patients, even when their plasma transferrin is far from saturation. A possible interpretation of this data is that, in hemochromatosis, iron enters the plasma from the gut in a NTBI form that is rapidly cleared by the liver and does not bind quickly to any transferrin available. Thus, the concentration of Bleomycin-detectable iron (NTBI) in plasma at anytime would presumably reflect a balance between entry of iron from the gut and its clearance by the liver (Aruoma, et al. 1988). Lehotay, D. et al (2001) have related the elevated NTBI only to hemochromatosis patients, and not other patients with elevated iron status parameters. We have tested plasma of patients severely affected patients with HH for congenital defects of glycosylation (CDG) syndrome by evaluation of immunoreactive forms of transferrin after IEF (Isoelectric Focussing) in polyacrylamide gels. The test revealed the absence of N-glycosylation defects associated with sialic acid deficiency. As is already known, carbohydrate–deficient Trf (CDT) plays an important role as a marker of chronic abuse and of congenital disorders of glycosylation such as COPD and cystic fibrosis. Hence, the defect we are suggesting in the transferrin molecule due to HH seems more likely to result from oxidative modification than N-glycosylation disorders.

7.4.1 Does transferrin–bound iron affect lipid peroxidation?

Cutteridge, J. et al. (1981) have saturated both lactoferrin and transferrin with iron to investigate the effect of partially iron-loaded transferrin on lipid peroxidation. They suggested that lactoferrin and transferrin, at physiological degrees of iron saturation, are inhibitors of

iron-dependent lipid peroxidation. This is due to their iron-binding capacities, since saturation of the proteins with iron abolished their inhibitory effect and indeed, might have caused them to have a pro-oxidant effect.

Cutteridge, et al. (1985) have differentiated between transferrin-bound iron and NTBI. As indicated above, they suggested that transferrin-bound iron inhibits lipid peroxidation. Most recently, they indicated that sera from patients with idiopathic hemochromatosis often contain iron detectable by the bleomycin method. They also suggested that a total serum iron greater than 40 μM is associated with the presence of bleomycin-detectable iron. According to Cutteridge et al. (1985), in at least one patient, the bleomycin-detectable iron was capable of stimulating both the peroxidation of lipids and the formation of the highly destructive hydroxyl radical. The transferrin in this patient's serum was essentially fully iron-saturated. In normal sera or partially iron-loaded transferrin sera, no iron can be detected by the bleomycin method. This observation might explain why we detected transferrin modification in samples of plasma from certain patients with HH while it was not detectable in other patients with the same disease. As indicated in Table 3, it seems that the patients with iron > 40 μM were prone to develop oxidative injury due to the full saturation of transferrin with iron. Hence, the iron found in the sera or plasma of iron-overloaded patients with idiopathic hemochromatosis is capable of stimulating oxygen radical reactions, which could contribute to the pathology of the disease.

7.4.2 Is Iron-loaded Transferrin able to generate ·OH?

As known, transferrin is the iron-transport protein of the human plasma. In the presence of a suitable anion (carbonate) it binds 2 molecules of Fe (III)/mol of protein. There is a dispute in the literature concerning the ability of transferrin-bound iron to accelerate ·OH formation and lipid peroxidation. McCord and Day (1978) reported that iron-loaded transferrin promoted ·OH

formation from superoxide and hydrogen peroxide at pH 7.4. This observation has been supported by others. Baldwin et al (1984) have indicated that iron-loaded transferrin was not active in accelerating ·OH formation at pH 7.8. Halliwell et al. (1985) have reported that ferric ions might become released from transferrin at low pH values (e.g. in ischemic tissues or in the microenvironment of activated phagocytic cells) in a form able to promote radical reactions, since iron can be mobilized from the protein under acidic conditions.

We suggest that transferrin-bound iron *in vitro* and *in vivo* is not active in accelerating oxidative damage at physiological pH unless a strong chelating agent is present. We have attempted to address this possibility in our first set of *in vitro* oxidation experiments with ferritin and transferrin by using hydrogen peroxide and EDTA as a chelating agent in physiological PBS at pH 7.4. The outcome was a damage of both proteins leading to their autoxidation, with several peptides containing oxidized methionine, tryptophan and histidine being detected. Furthermore, in our second set of *in vitro* oxidation experiments with transferrin, in the absence of EDTA, we achieved even greater damage of the transferrin molecule in the presence of carbonate and ferric nitrilotriacetate at an acidic pH which promoted the radical reactions. It seems that pH as well as chelating agents are playing an important role in initiating oxidative injury.

7.5 Methionine Oxidation

7.5.1 Significance of In Vitro Met Oxidation

Methionine is one of the most readily oxidized amino acid residues of proteins. It is attacked by hydrogen peroxide, hydroxyl radical, hypochlorite and peroxynitrite. Under the effect of methionine sulfoxide reductase, methionine sulfoxide (the initial methionine oxidation product) can be reduced back to methionine. However, a higher concentration of hydrogen peroxide or any other oxidant will lead directly to the production of the irreversible oxidation product, methionine sulfone, without intermediate generation of hydroxyl radicals (Vogt, W. 1995). Functional changes by Met oxidation in a given protein appear to have pathophysiological significance in some diseases such as rheumatoid arthritis, emphysema and, recently, hereditary hemochromatosis in which Met is converted into Met sulfoxide. Considering the reversibility of Met oxidation in some instances, it seems possible that Met oxidation/reduction in proteins may be involved in maintaining homeostasis in biological systems. Met sulfoxide can be formed as a Met oxidation product *in vivo* but formation of sulfone (irreversible oxidation product) is unlikely, although this is possible via *in vitro* experiments since we can control the strength of the oxidizing agent.

Based on these considerations, the development of a disease due to Met oxidation is most likely due to accumulation of Met oxidation products and/or the involvement of another oxidation marker rather than dependence upon the production of irreversible oxidation product (sulfone) *in vivo,* which as we have described, is unlikely.

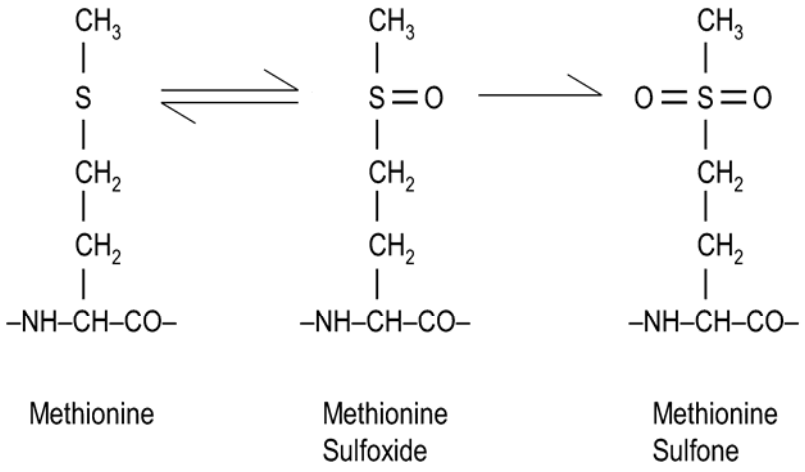


Fig.21: oxidation products of Methionine

Oxidation of up to 10% of the C-terminal methionine residues in proteins can occur spontaneously. The best-known methionine-selective chemical oxidants are chloramines T (Cl-T) and *N*-chloro-succinimide (*N*-Cl-S). Cl-T is a relatively mild oxidant that, at pH 7.0 or higher, oxidizes only cysteine and methionine residues, whereas *N*-Cl-S also attacks tryptophan. An extremely specific oxidant for methionine residues is dimethyl sulfoxide. Under proper conditions it leaves even cysteine intact (Vogt, 1995).

Methionine can be oxidized by biologically relevant oxidants such as superoxide, hydrogen peroxide, hydroxyl radical and hypochlorous acid which are products of PMN (polymorphonuclear neutrophils) leukocytes and other phagocytic cells used to damage invading organisms in defence reactions of the host (Vogt, 1995). In our methods, we have used one of these biologically relevant oxidants (hydrogen peroxide) to achieve a better matching for *in vivo* future findings. With the exception of superoxide, all such oxidants can oxidize methionine to the sulfoxide. Furthermore, methionine was extensively oxidized, yielding the highest scoring modified peptides for ferritin and transferrin in both *in vivo*

and *in vitro* work. We suggest that, provided catalytic amounts of iron are present, rather low concentrations of hydrogen peroxide are sufficient to oxidize methionine. In the first portion of our *in vitro* work, we obtained an abundance of modified methionine residues in the presence of EDTA (iron chelator), which is known to enhance protein autoxidation.

In the second portion of our *in vitro* work, we obtained even higher scores for oxidatively modified methionine residues in the absence of EDTA. This underscores the role of transition metals (iron or copper) as catalysts for the decomposition of the hydrogen peroxide-producing hydroxyl radical in the course of tissue damage or oxidative stress.

7.5.1.1 Inhibition of Met Oxidation

For *In vitro* studies, scavengers of oxidants are used for either of two purposes: (1) to stop the reaction of the oxidants with proteins (specifically with methionine); or (2) in the case of complex biological systems, to obtain some indications on the nature of the active oxidant leading to the effect observed by Vogt (1995). The first case is most applicable to our experiments since, specificity is not required; any agent that reacts with the oxidant but does not interfere with the product or its estimation can do the work. Ethanol, mannitol, and dimethyl sulfoxide have all been used as scavengers for hydroxyl radicals. Iron chelators, such as EDTA, can be used for abrogation of further production of hydroxyl radicals (reported in our Materials and Methods Section).

7.5.2 Is there a relationship between methionine and tryptophan oxidation?

The detection of both Met and Trp oxidation in both our *in vitro* and *in vivo* experiments requires more detailed discussion.

Taylor, S et al. (2003) assumed Trp oxidation to be analogous to Met oxidation, known to be an artifact of sample handling, since there is no correlation between Trp oxidation and the Met oxidation, thereby suggesting they occur via different mechanisms. In other words, the method of preparation is not a factor in determining the extent of oxidation or the presence of oxidation in one sample and its absence in another sample.

Taylor et al. reported that Met oxidation appears to be related directly to the number of observable peptides, as would be expected if oxidation were a random sample-handling artifact. In contrast, Trp oxidation appeared much more specific to selected subunits.

In our *in vitro* experiments we have detected Met and Trp as well as His oxidative modifications. Met is the most susceptible amino acid for oxidation by several oxidants such as hydrogen peroxide, our oxidizing agent of choice. Furthermore, the severity of oxidation varied between iron release and iron incorporation models since, we detected more oxidized Met residues among those samples with iron incorporation compared to those with iron release. Of course, random sample-handling artifact will never differentiate between both preparations. Furthermore, the absence of gel separation in our protocol reduces the risk of random Met oxidation. Taylor's observations that Met and Trp oxidations are not correlated further strengthens our hypothesis that the observed Met oxidation is real . Our *in vivo* findings indicated the presence of oxidized Met peptides in plasma samples from certain patients with HH. These and Trp oxidations detected in other samples support our contention that Met oxidation is not artifacted.

7.6 Tryptophan Oxidative Modification as a Biomarker of Oxidative Stress

Metal-catalyzed oxidation (MCO) is a primary oxidizing system in many cell types. The oxidative effects of MCO (Fenton reaction) on the structure of tryptophan residues was extensively investigated. Finely, E. et al (1998) have investigated the structure of the tryptophan and methionine residues of bovine α-crystallinin eye lenses. Oxidation of proteins via the Fenton reaction depends on the ability of the protein to bind iron; that is, proteins with high affinity for iron are readily oxidized (Samuni et al. 1990; Stadtman, 1992). The presence of an EDTA-Fe complex prevents oxidation of proteins that binds iron (Stadtman, 1990, 1992).

The oxidation of tryptophan to *N*-formylkynurenine in proteins is well established. Taylor et al. (2003) have suggested that tryptophan residues are “hot spots“ for oxidation in respiring mitochondria. The most interesting aspect of tryptophan oxidation is its irreversibility as reported by Spector, A. et al. (1995) as he indicated its occurrence in cataractogenesis causing severe and irreversible opacity of the eye lenses. Finley, E. et al. 1998 have confirmed these findings. As we have mentioned above, tryptophan and methionine oxidations are not related. They might confirm the occurrence of oxidative stress. In our *in vivo* experiments, the occurrence of both oxidations gives support and strength to our hypothesis regarding the involvement of oxidative stress in altering the homeostasis of iron metabolism due to hereditary hemochromatosis that might be one of the cornerstones of the pathophysiology of this disease.

On page 97, we have detected a score of 7.83 % for OxW which was apparently low. However, this oxidative modification was repeated in different samples which might give more support for the effect of tryptophan oxidation in The pathology of HH.

Tryptophan oxidation has never been detected as an artefact or as a reversible condition. On

the other hand, methionine oxidation has been detected as an artifact, a reversible or an irreversible condition.

So, to indicate whatever a pathological condition is due to methionine oxidation we have to meet at least one of these 2 criteria: first, further investigations that will lead to the information that the oxidation we have is irreversible.

Second, using other markers for oxidative stress to confirm oxidative damage. In our experiments, we had 2 more markers, both were irreversible (protein carbonyls and Trp oxidation). However, Trp oxidation has been reported as an indication of cataractogenesis, whether the score of peptides containing oxidized tryptophan residues was high or not, simply because it has never been detected as a reversible condition or as an artefact, as mentioned above.

If in our *in vivo* experiments, we ONLY detected Met oxidation, that would never support the notion of having oxidative stress unless this data was supported with other data (as carbonyls or Trp oxidation). We suggest that Trp oxidation is most likely to be supportive. I think we cannot apply the conditions of the *in vitro* experiments TYPICALLY to the *in vivo* environment. However, the reason we have conducted the *in vitro* experiments prior to the *in vivo* ones was to target such modified peptides as a guide to identify the same in real (biological) samples rather than concerning about scores.

8.0 Conclusions

1. Ferritin and transferrin can be oxidatively modified *in vitro* by iron immobilization via an iron chelator (EDTA) OR iron incorporation.
2. Mapping of the oxidatively modified ferritin residues were shown to be situated on the inner face of each subunits (the face directed toward the ferritin core.
3. Hereditary Hemochromatosis is an ideal model for iron overload.
4. Fully Iron-saturated transferrin is a source of oxidative stress via low-molecular-weight iron and non-transferrin bound iron.
5. Methionine and tryptophan oxidations are confined to those plasma samples of HH patients who showed high transferrin saturation values and carbonyl content.

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